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CANADIAN THESES ON MICROFICHE | THÈSES CANADIENNES SUR MICROFICHE

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TITLE OF THESIS/*TITRE DE LA THÈSE* Anabaena flos-aquae Toxin : Its Toxicology and Mechanism of Action

UNIVERSITY/*UNIVERSITÉ* University of Alberta Edmonton

DEGREE FOR WHICH THESIS WAS PRESENTED/ *GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE* PhD

YEAR THIS DEGREE CONFERRED/*ANNÉE D'OBTENTION DE CE DEGRÉ* 1974

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Anabaena flos-aquae TOXIN: ITS TOXICOLOGY AND MECHANISM OF ACTION

by

© WAYNE WILLIAM CARMICHAEL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

ALGAL ECOLOGY

DEPARTMENT OF BOTANY

EDMONTON, ALBERTA

FALL, 1974

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled *Anabaena flos-aquae* TOXIN: ITS TOXICOLOGY AND MECHANISM OF ACTION submitted by WAYNE WILLIAM CARMICHAEL in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Algal Ecology.

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ABSTRACT

Outbreaks of blue-green alga poisonings are caused by a combination of genetic and environmental factors. Under certain environmental conditions, toxic strains of *Anabaena flos-aquae* (Lyngb.) de Bréb. become dominant in a waterbloom and cause significant amounts of an alkaloid toxin, called Very Fast Death Factor (VFDF) to accumulate. Death of livestock or other animals occurs if they consume enough of such a bloom to equal or exceed a critical dose. Previous work with toxic clone (NRC-44-1) from Burton Lake, Saskatchewan indicated that toxicity is regulated by growth of the alga. This same clone when obtained in the bacteria-free state was found to have a higher toxicity than its contaminated control. This thesis looks at the role that bacteria might have on clonal growth and toxicity. It also examines the variability of toxicity within colonies and between clones of the algae. It then examines the toxicology of the toxin using selected animal species. Finally it investigates the mechanism of action for the toxin using selected pharmacological tests.

Five bacterial isolates were found to affect growth of *An. flos-aquae* by means of cell lysis. Three of these have been identified as belonging to the genus *Pectobacterium* of the family Enterobacteraceae. Two isolates were found to affect toxicity of selected axenic clones by prevention of toxin production. One of these appears to be in the family Enterobacteraceae. The toxin-affecting bacteria can decrease optimal clone toxicity (minimum lethal dose (MLD) = 60 mg/kg IP mouse) to about 240 mg/kg body weight. MLD's greater than this

amount are accountable for by a mixture of toxic and non-toxic filaments within the culture derived from the same colony. Mixtures of axenic toxic and non-toxic clones have toxicities in proportion to the numbers of toxic and non-toxic filaments.

Calves, rats, ducks and goldfish given lethal oral doses of a bacteria-free lyophilized cell suspension of toxic *An. flos-aquae* died with symptoms characteristic of respiratory arrest. In calves and rats artificial respiration restored normal heart rate, ECG and blood pressure. The oral MLD for calves was estimated at six to eight times the mouse IP MLD/kg; rats 25 times; ducks six times; and goldfish two times. Male mallard ducks given algae in suspension either orally or IP developed opisthotonus. Some tachyphylaxis was observed in the responses of most species studied.

In rats, a toxin extract from *An. flos-aquae* NRC-44-1 blocked responses of the anterior tibialis muscle to stimulation of the sciatic nerve. On rat phrenic nerve-hemidiaphragm, the extract blocked responses of the muscle to nerve stimulation. In ducks, responses of the gastrocnemius muscle to stimulation of the sciatic nerve were blocked and the muscle contracted. In chicks, the extract produced a typical depolarizing muscle relaxant syndrome. On the frog rectus muscle, toxin extract gave a contractile response which was qualitatively similar to acetylcholine. d-Tubocurarine shifted dose-response lines of acetylcholine or the extract to the right in a parallel manner. On the guinea-pig ileum, large doses of extract caused a contraction which could be abolished by hexamethonium without affecting responses to acetylcholine.

It was concluded that lyophilized cells and extracts of the toxic strain *An. flos-aquae* NRC-44-1 contain a material which has many of the characteristics of a depolarizing neuromuscular blocking agent and is rapidly absorbed by the oral route. The active fraction in a preparation of synthetic material showed similar toxicology and pharmacology to the lyophilized cells and extract.

A conferva that is indigenous and confined to the lakes has been produced in excessive quantities, so much as to render the water unwholesome.

It is I believe, Nodularia spumigena, allied to protococcus. Being very light, it floats on the water except during breezes, when it becomes diffused. Thus floating, it is wafted to the lee shores, and forming a thick scum like green oil paint, some two to six inches thick, and as thick and pasty as porridge, it is swallowed by cattle when drinking, especially such as suck their drink at the surface like horses. This acts poisonously, and rapidly causes death; symptoms-stupor and unconsciousness, falling and remaining quiet, as if asleep, unless touched, when convulsions come on, with head and neck drawn back by rigid spasm, which subsides before death. Time-sheep, from one to six or eight hours; horses, eight to twenty-four hours, dogs, four to five hours; pigs, three or four hours.

George Francis-1878

ACKNOWLEDGEMENTS

Since this study overlapped with different biological disciplines there were several people without whose help the research would have been difficult to complete. First I would thank once again my advisor, Dr. Paul R. Gorham for his continued help and support throughout the study. To Dr. David F. Biggs, Faculty of Pharmacy I extend particular thanks for assistance with the toxicology and pharmacology experiments. I would also thank Dr. Fred D. Cook, Department of Soil Science for making available his bacteria collection to my work and for direction in the identification of certain bacterial isolates.

I then thank Mr. Ted Germaine of the Surgical Medical Research Institute (SMRI), Mr. Jack Francis, University Farm and Dr. R. Christopherson, Department of Animal Science for cooperation and assistance with the calf experiments. I am grateful to Mr. Jim Thomsen and Mr. Joe Bouvier of Bioscience Animal Services for their extra efforts in arranging for the animals used in these studies. I also appreciate the cooperation of Dr. C. M. Grieve, Department of Animal Science, Dr. J. Holmes and Dr. R. E. Peter, Department of Zoology in making available various animals for study. Appreciation is also due to Mrs. K. Strausz, Provincial Toxicology Laboratory and Mr. Alan MaCauly, Department of Agriculture, Extension Service for reporting cases of suspected algal poisonings to me for further investigation. The laboratory assistance of Mr. Pat Hurdle, Department

of Botany is also appreciated. His control device for automatic monitoring of mass culture concentrations greatly expedited obtaining large amounts of cell material for toxicology and toxin extractions.

Finally I want to thank my wife Laurel for typing this manuscript and for her tactful criticism and comments on its contents.

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INTRODUCTION

Poisonings by certain species of freshwater blue-green algae have been an infrequent but repeated occurrence in several parts of the world. Investigations initiated because of economic losses of livestock and wildlife by poisonous freshwater blooms have led not only to discovery of the organisms involved but also their ecology and physiology. Field reports and laboratory experiments indicate that animals can only ingest enough toxic algae to cause sickness or death when an algal bloom is concentrated in a body of water, usually by the wind. These wind-concentrated scum-like blooms are generally repulsive to man so he avoids them. The result is that ingestion of sufficient quantities by humans to cause illness or death has seldom been reported (Dillenberg and Dehnel, 1960).

The freshwater planktonic blue-green *Anabaena flos-aquae* (Lyngb.) de Bréb. has been responsible for some of the most extreme cases of freshwater algae poisonings due to its high toxicity and the numbers of animals it has affected. The toxin has been found to be an alkaloid[1] (Stavric and Gorham, 1966; Huber, 1972) exotoxin termed the Very Fast Death Factor (VFDF) because it can kill mice in just a few minutes (Gorham, McLachlan, Hammer and Kim, 1964). Characteristic symptoms of the toxin following intraperitoneal (IP) injection in mice include a latent period of about one to two minutes. This is followed by a one-to-two minute period of tremors, dragging of hind

[1]Nitrogen containing bases that are generally of plant or animal origin.

legs and spasmodic convulsions. Death is preceded by convulsive contractions of thorax and gaping mouth and occurs in four to five minutes. Death is presumed due to respiratory failure (Gentile, 1971). Investigations of toxic *Anabaena* blooms have found that toxicities vary in degree and length from one outbreak to another (Rose, 1953; and Gorham, 1964) and within colony isolates from the same bloom (Gorham, *et al.*, 1964). Changes in species composition were initially thought to be responsible but records indicate that toxicity may vary without much change in species composition being apparent. The discovery that there were toxic and non-toxic strains (colonies) (Gorham, *et al.*, 1964) of *Anabaena flos-aquae* led these authors to suggest that an important factor in bloom toxicity was dominance by toxic colonies. Additional factors which have been found to affect the apparent toxicity of waterblooms includes: animal access, dosage intake, detoxification by adsorption to sediments, grazing by protozoa, toxic bacteria and the growth and development of toxic blooms in relation to light, temperature and aeration (Gorham, 1962; and Gorham, personal communication). Carmichael (1972) studied the effects of mineral nutrition, oxygen tension and interactions between alga and contaminating bacteria on toxicity of a toxic clone of *Anabaena flos-aquae*. Findings implied that mineral nutrition and oxygen tension did not effect toxicity unless growth was also affected. Bacteria-free cultures, however, showed improved toxicity over contaminated controls which indicated that at least under laboratory culture conditions, there were species

and strains of bacteria which could lower or reduce toxicity of the alga without affecting its growth.

In addition to the important problem of ecological and physiological factors that determine the level of toxicity in water-blooms of *Anabaena* there is the toxicology and pharmacology of the toxin and what, if anything, may be possible in the way of usable antidotes. For determinative toxicological studies of natural compounds it is important to isolate, identify, and culture the organism responsible. It is also important to obtain constant levels of toxin production from one culture batch to another. Finally, it is very important with microorganisms, such as the planktonic blue-green algae to obtain bacteria-free or axenic cultures to eliminate not only the possibility that bacteria are producing (either directly or indirectly) the toxin but also of any growth or toxin production effects that they may have on the algae. Pharmacological investigations ideally require that the toxic compound be isolated and purified. However, a certain amount of useful information can be obtained with fractions in which the toxin has been concentrated but ultimately it must be pure for critical investigations. It is desirable, if possible, to prove any proposed structure of a toxin by partial or total synthesis.

This study of toxicity in *An. flos-aquae* was undertaken with the following objectives:

1. To isolate, culture and identify bacteria from laboratory cultures and natural blooms of *Anabaena* or other blue-green algae and test their effects upon growth and toxicity of an axenic clone of *Anabaena*.

2. To determine whether toxin reduction by such bacteria is caused by degradation of the toxin or inhibition of toxin production by the alga.

3. To investigate which of three possibilities or combination thereof is responsible for the observed variability in toxicity of *Anabaena* colony isolates cultured under standard conditions in the laboratory and to interpret the results in relation to the variable toxicity of *Anabaena* waterblooms in nature. The three possibilities are:

(a) bacteria may be directly or indirectly responsible for the range of toxicity levels recorded,

(b) a colony may be a mixture of toxic and non-toxic filaments, or

(c) a colony may be a mixture of filaments with genetically different toxicities.

4. To describe the toxicology in greater detail by oral and intraperitoneal (IP) dosings of toxin into animals that have been most frequently killed by natural waterblooms of *Anabaena*. These tests are to provide dosage levels and symptoms associated with sickness or death for practical use in helping to identify *Anabaena* poisonings in the field and in helping to understand the pharmacological or physiological mode of action of the toxin.

5. To describe the pharmacology of the toxin using highly concentrated extracts or fractions. This involves the use of selected animal tissue preparations and the testing of a number of possible pharmacological antagonists for the toxin.

LITERATURE REVIEW

A. Freshwater Blue-green Algae Responsible for Toxic Blooms

This thesis is an extension of a recent study on toxic *Anabaena flos-aquae* (Carmichael, 1972) in which work on other toxic blue-greens has been reviewed. Gentile (1971) has provided a recent review which describes much of what is known about the structure and pharmacology of the blue-green toxins. Of more than 50 genera and 250 species of freshwater blue-greens, only six genera are considered toxic. Of these six genera, only three, *Microcystis*, *Aphanizomenon* and *Anabaena*, have been extensively studied in the laboratory. Within these genera toxic and non-toxic strains have been isolated, so far, from one species of each: *Microcystis aeruginosa* Kütz. emend. Elenkin, *Aphanizomenon flos-aquae* (L.) Ralfs. and *Anabaena flos-aquae* (Lyngb.) de Bréb. (= *Anabaena Lemmermannii*). *Microcystis* toxin is a cyclopolypeptide (Bishop, Anet and Gorham, 1959). *Aphanizomenon* toxin is an alkaloid reported to have a structure similar to that of saxitoxin or shellfish poison (Jakim and Gentile, 1968) and *Anabaena* toxin is an alkaloid (Stavric and Gorham, 1966). The remaining literature review will be centered on field and laboratory studies of *Anabaena flos-aquae* and its toxicology.

B. Occurrence and Case Histories of Toxic Waterblooms of *Anabaena flos-aquae*

Most documented reports of toxic *Anabaena flos-aquae* have come from North America and are reviewed by Schwimmer and Schwimmer

(1968). Some selected cases of algae poisoning in which *Anabaena* has been at least a component of the bloom include those described by Fitch, Bishop and Boyd (1934), Stewart, Barnum and Henderson (1950), Rose (1953), Dillenberg and Dehnel (1960) and Olson (1964). The species composition and symptoms described in these reports are summarized in Table 8. These blooms were held responsible for the deaths mainly of cows and ducks. Dosing of rabbits, pigeons, cows and mice with samples of waterbloom algae and water in the laboratory provided symptoms and lethal dosages as well as evidence that algae were the responsible organisms. *Anabaena* was given as the dominant organism only in the cases cited by Rose and Olson. In the cases reported by Fitch, *et al.* and Stewart, *et al.*, *Anabaena* sp. was only a small component of the bloom. There were, however, descriptions of of symptoms which were common to all poisonings among birds (legs rigid, neck stiff and contracted). It is possible that the *Anabaena* component was responsible for the deaths and symptoms reported but it is also possible that one or more of the other species of blue-green algae that were present may have been partially or wholly responsible for poisonings that were very *Anabaena*-like.

Two recent cases in Alberta which the author had an opportunity to investigate will be documented here:

Case 1. Disney Lake near Strathmore, Alberta, June 1972. A sample of waterbloom from this lake collected on June 22 was submitted to this laboratory by Mrs. K. Strausz of the Provincial Laboratory, Toxicology, O. S. Longman Building, Edmonton. The death of three calves which had

occurred two weeks earlier (Plate 1A) was attributed to a waterbloom present in this lake at this time (Strathmore Veterinary Clinic Pathology No. 72-3305). The June 22 sample consisted almost entirely of *An. flos-aquae* and had a concentration of 9.1 g/l dry weight of cell material. It had an MLD (IP mouse) of 480 mg/kg body weight and produced typical VFDF survival times and symptoms. The MLD of the bloom concentrate that was bioassayed would not have been sufficient to kill the calves considering the volume of water they would have had to drink (approximately 30 liters for a 60 kg calf assuming an oral MLD of 4,800 mg/kg, which is 10 X the IP MLD/kg mouse). Presumably the toxicity of the bloom had been much greater when the calves died two weeks earlier. No one observed them dying but death was estimated to have occurred one-half to three hours after the animals had access to the water. Fresh samples of lake water containing low concentrations of healthy colonies of *An. flos-aquae* were collected from Disney Lake by the author on June 25. Colony isolates from these samples grown in the laboratory were all toxic. Water samples collected at this time and submitted to the Provincial Laboratory for analysis showed the lake to be highly alkaline (total alkalinity equaled 688 ppm).

Case 2. Beaverhill Lake, near Mundare, Alberta (about 40 miles east of Edmonton), September, 1972. On September 3 cattle which had access to the lake (Plate 1B and 1C) began dying from suspected algae poisoning. Although cattle were removed from the water as a drinking source on September 4, cattle continued to die as late as September 6.

PLATE 1A. Calf dead from waterbloom of toxic *Anabaena flos-aquae* in Disney Lake (near Strathmore), Alberta. June, 1972.

PLATE 1B. Concentrated shoreline bloom of toxic *Anabaena flos-aquae* in Beaverhill Lake (east side) (near Mundare), Alberta. September, 1972.

PLATE 1C. Wind-concentrated bloom on shore.

In all, 15 head of cattle died and autopsies done on 13 of the 15 animals by Dr. A. Bidsell, Vegreville Veterinary Clinic indicated algae poisoning as the cause of death (Pathology no. 72-4273, September 5, 1972). Mice injected (IP) with waterbloom by the Veterinary Clinic at the same time as the cattle were dying had survival times of approximately one hour. *An. flos-aquae* was the only alga present in decomposing bloom samples submitted to this laboratory. Mice injected with aliquots (IP) had survival times of one to one and one-half hours but an MLD was not established. The symptoms observed did not resemble those characteristic of VFDF, nor of microcystin (Konst, *et al.*, 1965). Since survival times and symptoms for mice were not characteristic of *Anabaena* VFDF another toxic factor is implied. Colony isolates from samples collected during subsequent field trips to the bloom were made, and these too proved to be toxic as will be discussed later. Water samples from these later field trips were analyzed by the Provincial Laboratory and showed high total alkalinity (434 ppm).

C. Culture and Toxicity Studies of *Anabaena flos-aquae*

1. Culture Medium and Growth. A defined mineral medium for growth of a toxic colony isolate of *An. flos-aquae* (NRC-44) was developed by Gorham, *et al.* (1964). It was termed ASM-1 after ASM (Artificial Seawater McLachlan) developed for optimal growth of toxic *Microcystis aeruginosa* NRC-1 (McLachlan and Gorham, 1961). Later work by Peary and Gorham (1966) defined the optimal light intensity

and temperature for growth and toxicity of an axenic clonal derivative (NRC-44-1) of *An. flos-aquae*. These optimum conditions were used for mineral nutrition and oxygen tension studies on growth and toxicity of the same clone, but non-axenic, by Carmichael (1972). These studies were done to determine if mineral nutrient concentrations or great changes in oxygen tension such as occur in decomposing blooms could account for the variable toxicity observed in these blooms. In all these instances where environmental parameters were studied toxicity was found to increase only if growth increased and conversely if growth decreased.

2. Colony Isolates and Toxin Variability. Gorham, *et al.* (1964) found that toxicity varied considerably among unialgal colony isolates of *An. flos-aquae* from the same sample of bloom collected from a Saskatchewan lake for which a prior case of algal poisoning was on record. In addition toxicity varied between successive subcultures of the same colony isolate. It was to find the reasons for this variability that various growth and toxicity studies have subsequently been done. Understanding the variable toxicity of a unialgal colony isolate, it was thought, would go far towards understanding the variable toxicity of *An. flos-aquae* waterblooms.

3. Influence of Bacteria on Growth and Toxicity of *Anabaena flos-aquae*. Recent years have seen the publication of a number of important studies and reviews on the lytic effects of cyanophages (Padan and Shilo, 1973) and bacteria (Daft and Stewart, 1971 and

1973; Shilo, 1971a; Stewart and Brown, 1971) on blue-green algae. In addition to these detrimental effects on growth it has been suggested that under certain conditions bacteria can stimulate algal growth by making available an increase in CO_2, derived from respiration, in waters having a high biological oxygen demand (Lang, 1971). Although no systematic work had been done with bacterial effects on toxicity of blue-green algae some preliminary data have been reported which indicate that bacterial contaminants reduced the toxicity of an axenic clone of toxic *An. flos-aquae* (Carmichael, 1972). Elimination of bacteria from clone number NRC-44-1 caused almost a doubling of toxicity without affecting growth. This suggested the possibility that during and after the development of a waterbloom one or more types of bacteria may be present which cause the variable toxicity of blooms. This hypothesis could be tested in the laboratory by making many bacteria isolates from a number of waterblooms and measuring what effect they had upon the toxicity of axenic *An. flos-aquae* NRC-44-1 in combined culture.

D. Toxicology and Pharmacology of Naturally Occurring Toxins

1. General. Plants have historically received a great amount of attention and research due to the great numbers that have some physiological effect on animals. These effects are varied and depending on the drug or the dose involved it is often said that the animal has been poisoned. It was to study the special medicinal effects

or direct poisonings caused by plant products that the science of pharmacognosy developed. This science encompasses those phases of knowledge relating to natural products which are generally of medicinal value and primarily of plant origin. Input to this science has come from botanists, chemists, pharmacologists and other related specialists. Examples of important contributions from this area of science include; quinine, the antimalarial from the chinchona tree; digitalis, from foxglove, which is a cardiac glycoside effective in treating congestive heart failure; corticosteroids from Mexican yams and curare muscle relaxants from moonseed liana or *Chondodendron toxicoferum*. These are but a few examples of natural plant products effective in various medical problems. An excellent historical review of these natural plant products is found in Krieg (1965).

Natural products from plant sources which, in certain cases, are poisons do not have to possess medicinal or clinical benefits to be of use to man. In recent years the study of their chemical identification and mechanism of action has helped greatly to understand physiological mechanisms in man particularly neuromuscular transmission (Bowman, 1973). The relative toxicities of many natural toxins, both plant and animal, vary greatly as indicated in Table 1.

The large area of microbial toxins is of particular interest to this study and is covered in an extensive eight-volume review. The algal toxins are reviewed in Volume 7 (Kadis, Ciegler and Ajl,

TABLE 1

Relative toxicities and other properties of a selected group of naturally occurring toxins.[1]

Toxin	MLD (µg/kg)	Chemical Class	Molecular Weight
Botulinus toxin A	0.00003	Protein	900,000
Tetanus toxin	0.0001	Protein	100,000
Ricin (castor bean)	0.02	Protein	-------
Saxitoxin	9	Alkaloid	372
Tarichatoxin (Californian newt)	8	Alkaloid	319
Tetrodotoxin (Pufferfish)	8-20	Alkaloid	319
Cobra neurotoxin	20	Protein	-------
Crotalus toxin (rattlesnake)	60	Protein	-------
Aphanizomenon toxin	50-100	--------	300
Microcystis FDF	100	Cyclic polypeptide	2,600
Anabaena VFDF	250	Alkaloid	300
Curare	500	Alkaloid	696
Strychnine	500	Alkaloid	334
Muscarine	1,100	Alkaloid	210
Glenodine toxin (Dinoflagellate)	2,500	Alkaloid	-------
Sodium cyanide	10,000	Organic salt	-------

[1]Adapted from Gentile (1971).

1971). The microbial toxins include those of bacterial, fungal and algal origin. Most of the toxins have remained under the heading plant poisons or infections although fungal toxins have important antibiotic benefits.

Some freshwater and marine algal toxins, including those from certain dinoflagellates (saxitoxin) and *Aphanizomenon flos-aquae* toxin (saxitoxin-like) have neuromuscular effects. In terms of mechanism of action, these toxins fall in the same group as curare, tetrodotoxin (pufferfish), bungarotoxins (snakes) and others. It was to this area of work that the mechanism of action for *An. flos-aquae* ultimately led and it is therefore necessary to say more about these neuromuscular toxins.

2. Neuromuscular Blocking Agents from Natural Sources. Probably the best review of neurohumoral mechanisms and neuromuscular blocking agents is found in the two-volume series on neuromuscular blocking and stimulatory agents (Cheymol, 1972). Handbooks such as Goodman and Gilman (1970) also provide much information on the subject. In addition, any textbook on comparative animal physiology (*e.g.* Prosser, 1973), or selected reviews on individual blocking or stimulatory agents provide information on neuromuscular blocking agents.

The concept of neurohumoral transmission holds that nerve impulses elicit responses in smooth, cardiac and skeletal muscles, exocrine glands and post-synaptic neurons through liberation of

specific chemical substances. The divisions of the peripheral autonomic system that elicit these responses are the sympathetic and parasympathetic. The neurohumoral transmitter of all preganglionic autonomic, all postganglionic parasympathetic fibers and some postganglionic sympathetic fibers is acetylcholine (Ach). These fibers are referred to as cholinergic. The adrenergic fibers comprise most of the postganglionic sympathetic fibers and here the transmitter is norepinephrine or noradrenaline (Koelle, 1970a). In this study we are mainly concerned with postganglionic parasympathetic fibers and their connections or synapses with muscle fibers.

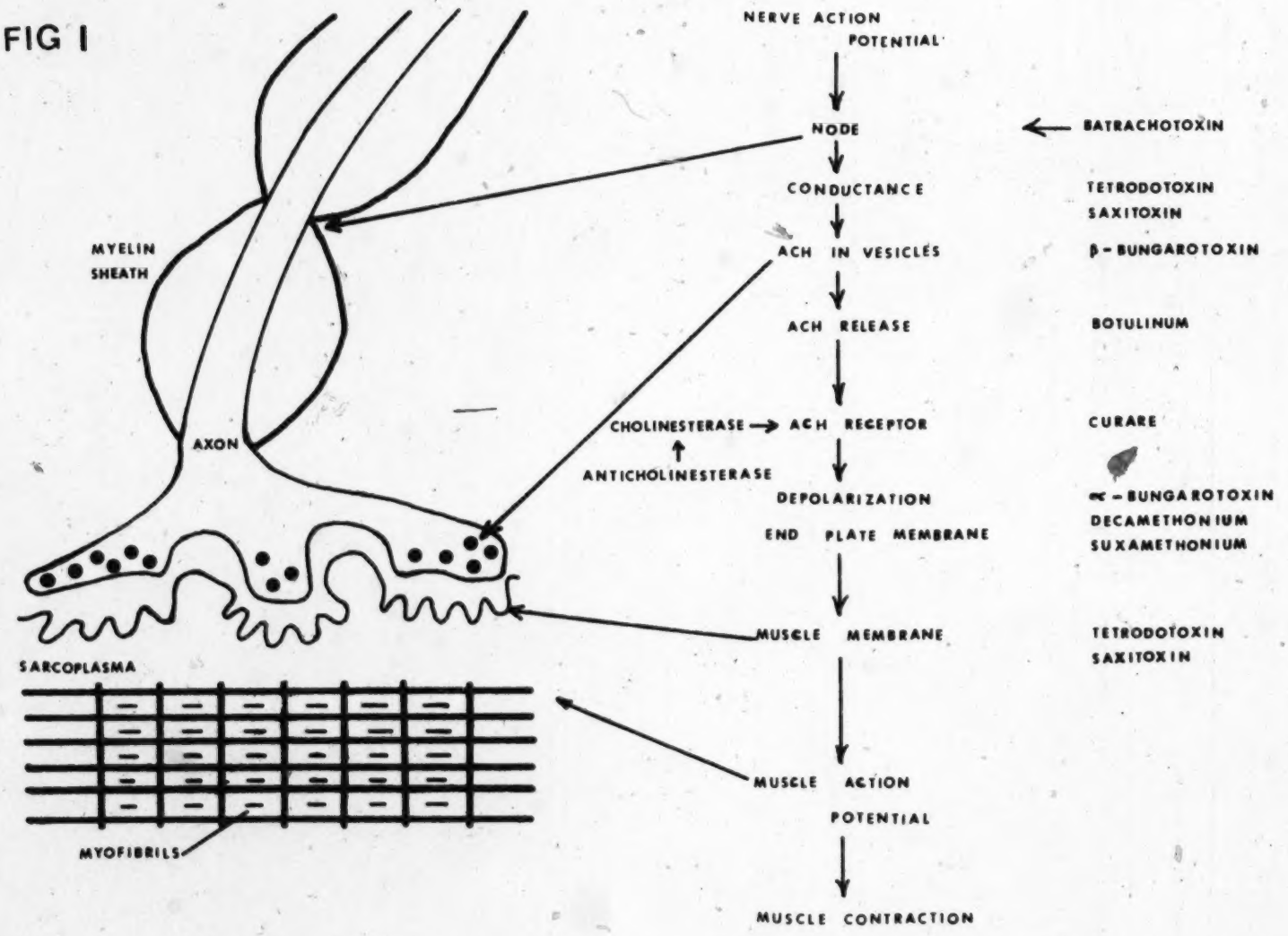
Neuromuscular blocking or stimulatory agents affect these cholinergic nerve fibers, muscle synapses and/or muscle fibers in various ways. The natural toxins which affect neuromuscular transmission occur in both animals and plants. The best way to describe the various mechanisms of action by these natural toxins is through a short description of what is known about them along with reference to a schematic diagram of a typical neuromuscular synapse (Figure 1).

Batrachotoxin is a steroidal alkaloid isolated from the skin of the Columbian poison arrow frog *Phyllobates aurotaenia*. It produces neuromuscular block by selective and irreversible increase of sodium permeability in the axon membrane at the nodes of Ranvier causing depolarization (Bowman, 1973) (Figure 1). Tetrodotoxin is an alkaloid obtained from the viscera of the genus *Fugu* (pufferfish). An alkaloid from the eggs and embryos of the Californian newt (*Taricha torosa*), originally called tarichatoxin, has been found to be identical in

FIGURE 1

Neuromuscular apparatus and toxins that affect it. Adapted from Koelle (1970b); Cheymol and Bourillet (1972a); and Bowman (1973).

FIG 1


NERVE ACTION POTENTIAL
NODE
CONDUCTANCE
ACH IN VESICLES
ACH RELEASE
CHOLINESTERASE
ACH RECEPTOR
ANTICHOLINESTERASE
DEPOLARIZATION
END PLATE MEMBRANE
MUSCLE MEMBRANE
MUSCLE ACTION POTENTIAL
MUSCLE CONTRACTION
BATRACHOTOXIN
TETRODOTOXIN
SAXITOXIN
β - BUNGAROTOXIN
BOTULINUM
CURARE
α - BUNGAROTOXIN
DECAMETHONIUM
SUXAMETHONIUM
TETRODOTOXIN
SAXITOXIN
MYELIN SHEATH
AXON
SARCOPLASMA
MYOFIBRILS

structure to tetrodotoxin. Saxitoxin is an alkaloid responsible for shellfish poisoning which is produced by two or more species of the marine dinoflagellate *Gonyaulax* (Cheymol and Bourillet, 1972b). Tetrodotoxin and saxitoxin have their effects at both sides of the neuromuscular synapse. They alter membrane permeability by blocking movement of sodium ions, essential for initiation of electrical activity. Several different snakes of the family Elapidae produce the α and β-bungarotoxins (polypeptides). α-Bungarotoxin has its effect by disrupting the Ach-containing vesicles and thus depleting the nerve ending of Ach. β-Bungarotoxin combines with Ach receptors on the muscle side of the synaptic gap and depolarizes the membrane (Vital-Brazil, 1972). Botulinum toxin (polypeptide) from the bacterium *Clostridium botulinum* prevents the release of Ach to the presynaptic membrane (Burgen, 1972). Curare is a general name for plant alkaloids that have muscle relaxing properties and come from various species of the genus *Chondodendron* or *Strychnos*. Its action is to prevent Ach attachment and thus it has a non-depolarizing blockade effect on the postsynaptic membrane (Waser, 1972).

The list just given does not include all the natural products which have neuromuscular effects, but it does include examples of most of the ways in which toxins are known to alter neuromuscular transmission. Other marine algal toxins include the dinoflagellate *Gymnodinium breve* (Schantz, 1971) which kills by respiratory arrest and seems to exert its effect through stimulation of postganglionic cholinergic nerve fibers (Grunfeld and Spiegelstein, 1974) and certain

Prymnesium species of the family Chrysophyceae. *Prymnesium* toxin is a lipoprotein with two toxic components, one hemolytic and the other having ichthyotoxic activity (Shilo, 1971). The toxin from *Aphanizomenon flos-aquae* has been proposed to be identical to saxitoxin (Gentile, 1971; and Alam, Ikawa, Sasner and Sawyer, 1973).

MATERIALS AND METHODS

A. Sources of *Anabaena flos-aquae* and Bacteria

An. flos-aquae isolates used in the experiments were either colonies, portions of colonies or individual clones (single filament) from a colony. Pre-existing laboratory cultures of this type included NRC-44-1 (Plate 2A and 2B) which is a clonal isolate from NRC-44 (Gorham, *et al.*, 1964). Colony isolates and most of the clonal isolates were from Disney Lake water samples (Plate 2C and 2D) and Beaverhill Lake. Disney Lake (formerly Bruce Lake) is located near Strathmore, Alberta at 52°11' N latitude, 113°32' longitude and in Tp 24-25, R 26. Beaverhill Lake is located near Mundare, Alberta at 53°27' N latitude, 112°32' longitude and in Tp 51-52, R 17-18. The colony isolates from Alberta-collected water samples were given the letter prefix "A" (Alberta) followed by a number. To designate isolates from these colonies the following coding system was adopted. Numerals following the original isolation number indicate filament isolates (clones) made from the original colony culture. Those that were multifilament isolates are indicated by a bracketed (m) at the end, otherwise they are single filament (clonal) isolates. Further isolations from these single or multifilament isolates are designated by an alphabetic sequence. Isolations from these isolates were given a numeral sequence, *etc.*

Bacteria types used were isolated mainly from laboratory cultures of toxic and non-toxic *An. flos-aquae* and from waterblooms of blue-green algae. Some bacteria were isolated from water samples collected after a toxic *An. flos-aquae* waterbloom had occurred (Disney

PLATE 2A. Freeze fracture *Anabaena flos-aquae* NRC-44-1 (X 11,400). Note numerous gas vesicles in cross section. Scale provided by gas vesicles which are about 50 to 60 nm in diameter (Walsby, 1973). These are important for buoyancy maintenance and bringing filaments to surface where they concentrate and become accessible to animals.

PLATE 2B. *Anabaena flos-aquae* NRC-44-1 (Nomarski interference contrast). Note heterocyst in middle of right filament.

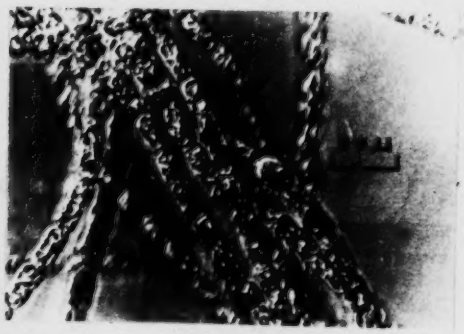
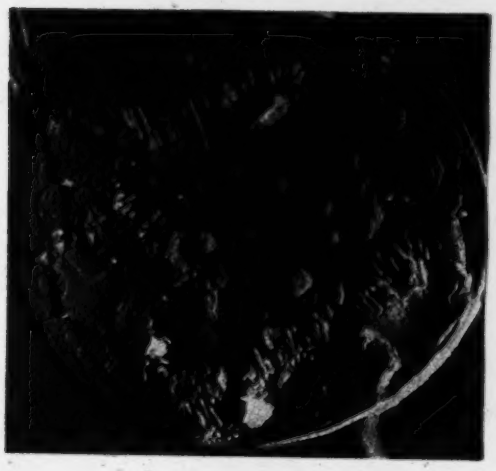
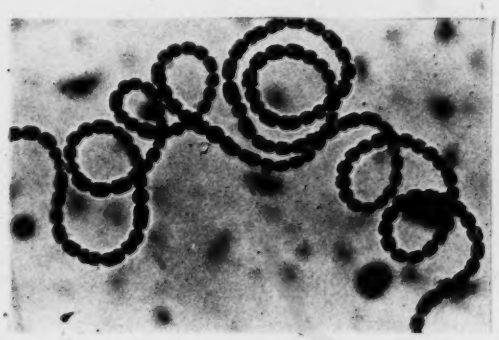


PLATE 2C. Freeze fracture *Anabaena flos-aquae* A-70 (X 16,200). Note gas vesicles (GV), photosynthetic thylakoids (Th) and polyphosphate granules (PG). Individual vesicle is about 200 to 300 nm long (Walsby, 1973).

PLATE 2D. *Anabaena flos-aquae* A-52 (phase contrast) (X 1,200).





Lake, June 25) and from sediment samples from Lake Wabamun located about 35 miles west of Edmonton. In addition 20 bacteria of known identity provided by Dr. F. D. Cook, Department of Soil Science, were tested.

B. Culture Conditions

1. *Anabaena flos-aquae*. Culturing of *Anabaena flos-aquae* was similar to earlier studies (Carmichael, 1972) which in turn were in part the result of work by Gorham, *et al.* (1964) and Peary and Gorham (1966). Inoculum cultures were grown in 50-ml Delong flasks containing approximately 30 ml of ASM-1-Tricine (ASM-1-TR) (Appendix 1 and 2) for about 10 to 14 days before transfer to fresh media. Test cultures for toxicity were grown in modified one-liter Delong flasks equipped for aseptic aeration (Plate 3A) which contained 600 ml of ASM-1-TR. Inoculum amounts were one per cent v/v from 10 to 14 day old cultures of the 50-ml inoculum flasks. Optimal growth was obtained using aerated cultures but it was also necessary to maintain aseptic conditions. This was accomplished by passing room air through sterile Whatman filters containing a no. 80 filter cylinder. This removed most room dust or other particles greater than 8 microns. Air then moved through two parallel sterile glass Bellco filters packed with 10 g of glass wool. Air was passed to individual cultures from two sets of glass manifolds containing 10 outlets apiece. Air flow was regulated by using Reciprotor piston pumps attached to a Variac. Air flow to individual cultures was controlled by screw clamps. The filter system was dry-sterilized monthly (sooner, if necessary) and

PLATE 3A. Modified 1-liter Delong flasks used in aseptic culturing of algae for bacteria and clone toxicity tests. Note air-filtering system to left.

PLATE 3B. Mass culture apparatus for large scale culturing of toxic clones.

the number 80 filter cylinder replaced. Flow rates of air to both modified Delong flasks and mass culture bottles were 1 to 1.5 l/min.

Mass cultures of *Anabaena* were grown in 10-liter Pyrex glass bottles fitted with fritted glass tube aerators and rubber stoppers (Plate 3B). Filtered air was passed through a similar system as for the modified Delong flasks with air being passed to cultures from a copper tubing manifold. Inoculum for mass cultures was one per cent v/v axenic *An. flos-aquae* NRC-44-1. All algae cultures were grown at 22 ± 1°C under continuous shaking, except for mass culture bottles which used air for mixing. Continuous illumination of 90 $\mu E/m^2/sec$[1] (5,000 lux), was provided from overhead cool white fluorescent lamps.

The rotary shakers used for the investigation were the same as previously used (Carmichael, 1972) and consisted of smaller units operated at 80 to 100 rpm, used for inoculum flasks, and a larger New Brunswick, model G-10, run at 60 to 80 rpm, used for the one-liter modified Delong flasks.

2. Bacteria. Water samples for bacteria isolations were collected from the field under the following aseptic conditions: water or algae bloom samples collected in sterile bottles had aliquots transferred to sterile medium by a flame-sterilized platinum needle. The medium used in the field was liquid plate count agar

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[1]Measured at the level of the culture surface with a Lambda L185 radiometer using an LI-190S quantum sensor or a LI-210S photometer sensor, respectively.

and skim milk medium (Appendix 3). Plate counts done in the field used sterile ASM-1-TR as the serial dilution media and 100x15 mm Petri dishes containing plate count agar. These samples were transferred to the laboratory for growth, isolation and testing. For certain field samples (decomposing blooms) additional media were used for streaking including ascorbic acid agar, maize agar, casitone agar, Lochhead's soil extract semi-solid medium and gelatin (Appendix 3). Bacteria isolated from laboratory cultures were streaked on plates of plate count agar and skim milk agar. All bacteria were incubated initially at room temperature. After isolation of individual bacteria colonies duplicate cultures were kept in a 10°C incubator (plates) and at 22°C (liquid in tubes). Viable bacteria counts were made, doing serial dilutions in duplicate, with ASM-1-TR as the dilution medium, for concentrations of 10^{-3}, 10^{-5} and 10^{-7}.

C. Colony Isolations

1. *Anabaena flos-aquae*. Colony isolates from field samples were isolated by the following procedure using a Leitz inverted microscope at a magnification of 80 X:

1. An individual colony or piece of a colony was placed on a slide in a drop of sterile ASM-1-TR.
2. Gentle blowing on the colony from a Pasteur pipette was used to break up the colony.

3. A piece of the colony was transferred to another drop of ASM-1-TR by capillary action of a Pasteur pipette.

4. Steps 2 and 3 were repeated six or seven times until a piece of colony was obtained that appeared free of protozoa or other algae (particularly diatoms). The clean colony was transferred to a test tube containing sterile ASM-1-TR, and then cultured under continuous illumination at 22°C. Axenic (bacteria-free) clones were obtained from these colony isolates by the method of Carmichael and Gorham (1974).

2. Bacteria. Bacterial isolates from natural or laboratory sources were made by streaking on plate count and skim milk agar plates. Individual colonies having distinct morphological differences were selected from these plates. This procedure was repeated as necessary until a single colony type was obtained. Individual colony types were kept on plate count and skim milk agar plates at 10°C and in test tubes, containing the same type of liquid media as the plates, at 22°C.

D. Growth Measurements

1. *Anabaena flos-aquae*. Algal growth was measured as biomass production based on optical density (OD) readings at 750 nm for time periods indicated by the individual experiments in the same manner as in previous work (Carmichael, 1972) except that the standard curve used was for aerated instead of non-aerated cultures. A stand-

ard curve relating OD 750 nm to biomass for aerated cultures of *Anabaena flos-aquae* NRC-44-1 is given in Figure 2.

2. Bacteria. Bacterial growth was measured as viable counts on plate count agar. A measurement of the amount of bacteria used for inoculations was obtained by doing serial dilutions in ASM-1-TR of the particular bacteria culture and determining the viable count. This was repeated at the end of an experiment to determine the increase or decrease in viable count.

E. Toxin Extraction Procedure

Toxin concentrates were required to get some idea of relative potency with other natural poisons and to allow toxicological and pharmacological experiments to be done on *in vitro* and *in vivo* animal tissue preparations. No entirely reliable technique exists for obtaining pure toxin extracts but highly concentrated ones were obtained using a method similar to Stavric and Gorham (1966). The starting material was a quantity (40 g in this work) of lyophylized cell culture of *An. flos-aquae* NRC-44-1. Quantities of this amount were obtained by the mass culturing technique mentioned earlier. Mass cultures were concentrated at 45°C on a continuously operating vacuum flash-evaporator. A flow chart for the extraction procedure is given in Figure 3. The presence of the toxin in an extract was verified before the mouse bioassay by its extinction peak at 229 nm. This was done using a Beckman DBG spectrophotometer and a UV hydrogen

FIGURE 2

OD (750 nm) versus biomass dry weight per liter, over the range from 0 to 1.0 OD units. Values were determined for aerated cultures of axenic *An. flos-aquae* NRC-44-1. The graph is also applicable to axenic *An. flos-aquae* A-52-2.

FIG 2

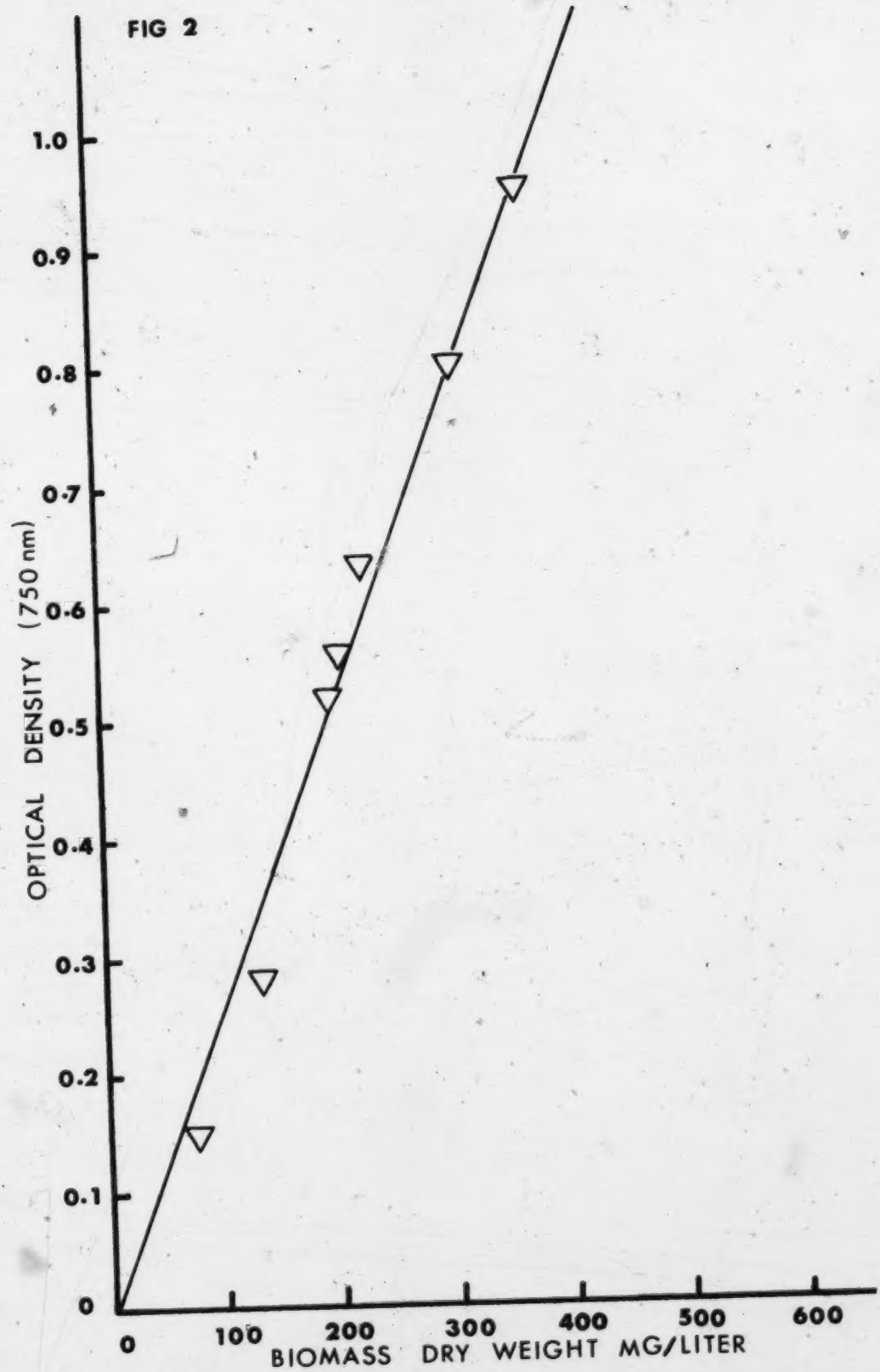
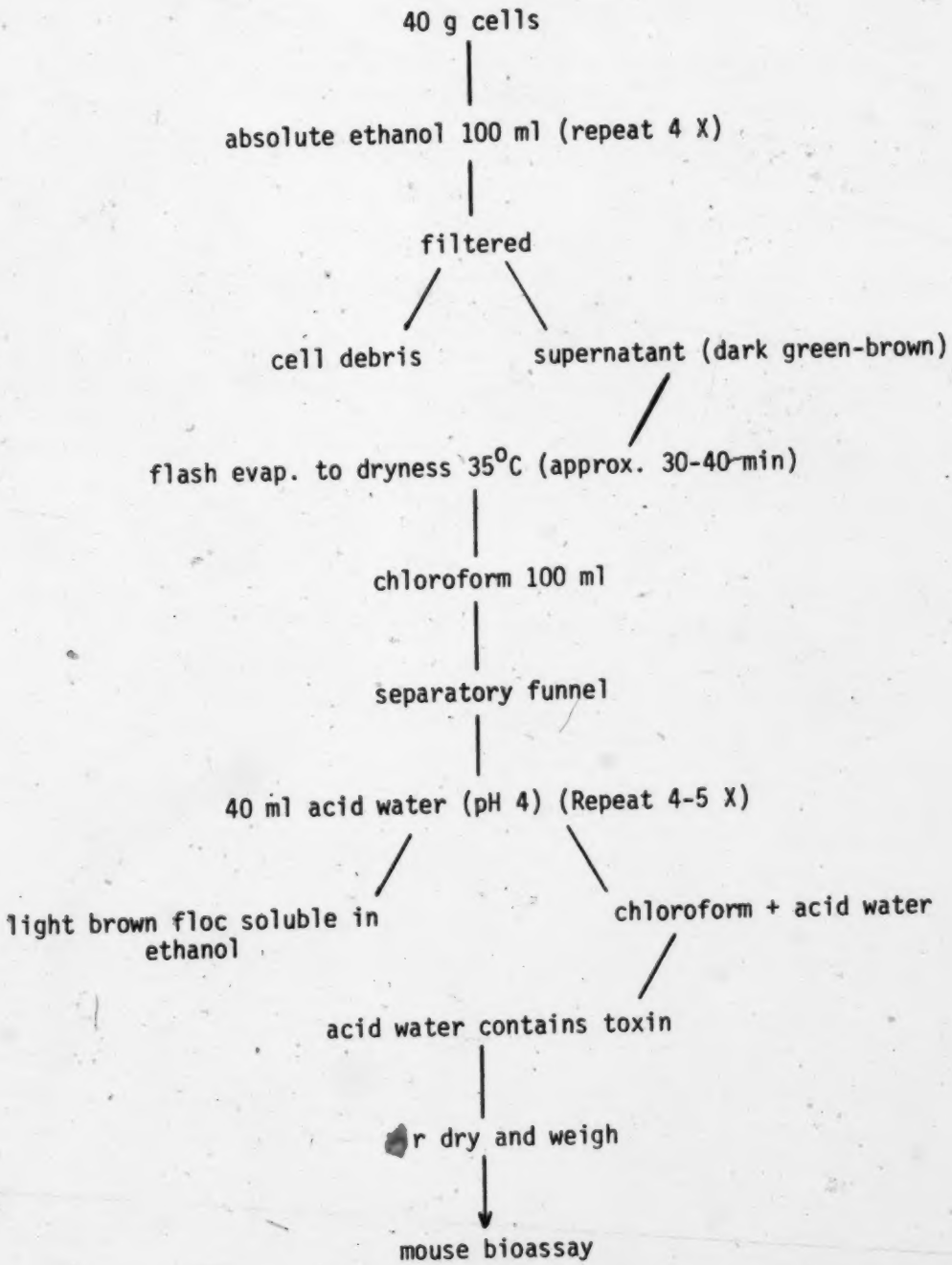

OPTICAL DENSITY (750 nm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
100
200
300
400
500
600
BIOMASS DRY WEIGHT MG/LITER

FIGURE 3

Toxin extraction flow diagram.

FIG 3


40 g cells
absolute ethanol 100 ml (repeat 4 X)
filtered
cell debris
supernatant (dark green-brown)
flash evap. to dryness 35°C (approx. 30-40 min)
chloroform 100 ml
separatory funnel
40 ml acid water (pH 4) (Repeat 4-5 X)
light brown floc soluble in ethanol
chloroform + acid water
acid water contains toxin
r dry and weigh
mouse bioassay

lamp power supply. Yields of concentrated extract by this procedure were adequate for the experiments conducted, and high in terms of total mouse units (MU)[1] recovered from the starting material. The 40 g of cell material with an MLD of 60 mg/kg (IP mouse) represented 26,667 MU of toxin. The total MU for the fractions collected by the extraction procedure was approximately 22,200 which is a yield of about 83%.

F. Basic Bioassay Procedure and Sources of Experimental Animals

Routine minimum lethal dose (MLD) or LD_{100} of the cell suspensions or toxin extract fractions were determined first using white male ALAS mice (15-25 g). The procedure was the same as that used by Carmichael (1972). Dosings were either intraperitoneal (IP) or oral as indicated for the individual experiment. The MLD (IP mouse) of a cell or extract fraction was then used as a basis for deciding upon the dosage levels that were used for other animals. The mouse bioassay was also used to test toxicity of all experimental cultures involving bacteria paired with algae and with clonal toxicity tests.

Most of the animals used in the experiments were obtained through the University Bioscience Animal Services. Mice and rats were from colonies maintained by Bioscience Animal Services. Calves were obtained from the Department of Animal Science with the cooperation of Dr. C. M. Grieve. Mallard ducks were obtained through

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[1]One MU = dose (IP) required to kill a 25 g mouse. An MLD represents 1000 g/25 g = 40 MU.

the courtesy of Dr. J. Holmes, Department of Zoology. Goldfish were obtained from Dr. R. E. Peter, Department of Zoology. Frogs and the guinea pig were obtained from Dr. D. F. Biggs, Faculty of Pharmacy. Chicks were obtained from Bioscience Animal Services.

G. Equipment Used for Pharmacology Experiments

Experiments in which respiration, blood pressure, heart rate and muscle contractions were monitored used the following equipment:

1. Respiration was monitored from a tracheal cannula connected to a Grass volumetric pressure transducer PT5-A.
2. Blood pressure was measured by a carotid or femoral artery cannula connected to a P23Dd (10 volt) transducer.
3. Output from these transducers was fed into a Hewlett Packard (HP) carrier amplifier 8805B.
4. Heart rate was computed from an 8812A rate computer connected to the carrier amplifier which received output from the blood pressure transducer.
5. The tendon or muscle from which muscle contractions were measured was string-connected to a Grass force displacement transducer FT 10 G.
6. All recordings were made on an HP-model 7754A 4-channel physiograph.

The muscle preparations for which this equipment was used are listed with the individual experiments and in Appendixes V to IX. Most of this equipment can be seen in Plate 7.

EXPERIMENTS AND RESULTS

A. Bacterial Effects on Growth and Toxicity

1. General. Bacteria were isolated from two main sources; lake waters and laboratory cultures (Table 2). Since the indication that bacteria lowered the toxicity of a laboratory culture had come from *Anabaena flos-aquae* NRC-44-1 which at one time had been axenic, became contaminated and then repurified, it was not clear whether other bacteria affecting growth and toxicity would be found in natural waterblooms or other sources of water. A total of 126 bacterial cultures were isolated from the two main sources and assigned isolation numbers as given in Table 2. In addition, 20 bacterial cultures of known identity were obtained from Dr. F. D. Cook, Department of Soil Science. It was very likely that some of these bacteria cultures were duplicates but no attempt was made to identify them unless they had some effect on growth or toxicity of an algal culture. Even if the number of bacteria isolates could be reduced by identification and elimination of species duplicates it could not be known what strain variability effects on the algae would be present.

To screen the numerous bacteria the following procedure was adopted:

1. Axenic cultures of *An. flos-aquae* were grown in one-liter modified Delong flasks containing 600 ml of ASM-1-TR. These cultures were inoculated at one per cent v/v from inoculum cultures grown in 50-ml

TABLE 2

Bacteria isolate numbers and their source.

Bacteria Isolate Numbers	Source
1 - 13	*An. flos-aquae* NRC-44-1 before repurification.
14 - 26	Astotin Lake. From *Aphanizomenon* and *An. flos-aquae* blooms.
27 - 46	Disney Lake. Isolates from sample of decomposing bloom of *An. flos-aquae* submitted by Provincial Toxicology Laboratory and from sample of lake water containing *An. flos-aquae* collected two weeks after water-bloom.
47 - 89	Beaverhill Lake. Samples from decomposing bloom of *An. flos-aquae*, bloom water and sediment under bloom.
90 - 115	*An. flos-aquae* cultures in laboratory both toxic and non-toxic.
116 - 126	Lake Wabamun. From sediment in outlet canal.
127	*Cytophaga brunescens* #2
128	*C. brunescens* #3
129	*C. brunescens* #5
130	*C. brunescens* #7
131	*C. brunescens* #9
132	*C. johnsonii* #405
133	*C.* sp. #460
134	*C.* sp. #446

Table 2 (cont.)

Bacteria Isolate Numbers	Source
135	*Cytophaga* sp. #497
136	*C.* sp. #18-H
137	*C.* sp. #15-D
138	*C.* sp. #444
139	*C.* sp. #463
140	*C.* sp. #17061
141	*Flexobacter canadensis* #9-D
142	*Lysobacter antibioticus* #A(1)-3C
143	*Lysobacter enzymogenes* #4721
144	*L. enzymogenes* #4740
145	*L. enzymogenes* #495
146	*L. enzymogenes* #13-B
147	*L. enzymogenes* #AL1

Delong flasks (non-aerated). These inoculum flasks were used when they were about 10 days old.

2. Initially, test cultures of algae were paired with bacteria after about 10 days when growth was in late log phase (or an OD (750 nm) of 0.6 to 1.0). Later, bacteria were paired with algae at an OD of 0.2 to 0.3 (6 to 7 days), and cultured until an OD of 0.6 to 0.7 was reached.

3. At pairing time, bacteria were added aseptically to the algae test culture to give an initial concentration of 10^4 to 10^5 viable cells per ml of algae culture. This concentration was obtained in 0.1 ml of bacteria culture and was of the order of that found in samples of lake and non-axenic laboratory cultures. The concentration of bacteria would be much higher in decomposing blooms (10^7 to 10^9 viable cells/ml).

4. The OD was recorded for the culture just after the bacteria were added and again after the four-day test period.

5. After four days, 300 ml of mixed culture were lyophilized and bioassayed for toxicity. Bacteria counts were also done to check for change in numbers over the test period.

Some bacteria lysed the algal culture, usually within 24 hours. In these cases, the test run was terminated and bacteria counts done. Culture facilities permitted a total of 20 modified Delong flasks to be cultured at one time. This capacity was divided so that 10 algae cultures had bacteria being tested while 10 more were being grown for testing. For each experiment conducted it usually required a minimum

of six mice to obtain an MLD. This was accomplished because the MLD of the algal culture at the start was well established on its OD 750 value. On the basis of six mice per test and allowing for the many duplicates eventually run, it took about 2,000 mice to complete this portion of the investigation.

2. Growth. Of the two ways that bacteria might be expected to affect growth, *i.e.* increase or decrease, the experimental tests were designed to select for growth decrease or lysis if it was to occur. By growing algae to their optimum growth stage before adding bacteria the only practical effect to be expected would be some degree of degradation. Growth could not be improved greatly because nutrients other than CO_2 would be limiting in the culture.

According to published work, lysis of blue-green algae by bacteria can be of two types. The first type is lysis by attachment to an algal filament and subsequent production of an unknown lytic compound (Daft and Stewart, 1973; and Shilo, 1971). The second type is by bacterial production of an extracellular enzyme (lysozyme-like) which causes algal lysis (Stewart and Brown, 1971). In all cases the bacteria responsible are non-fruiting *Cytophaga* of the Myxobacteriales. Lysis refers to dissolution of cell wall as in blue-greens whereas "killing" would refer to killing of green algae without lysis (Stewart and Brown, 1969).

To test the two types of bacteria effects it was necessary to pair algae and bacteria with both shaken and unshaken cultures. The unshaken cultures would give optimal test conditions for bacteria

that might require contact with the alga for lysis to occur. For stationary culture tests 50-ml Delong flasks (non-aerated) were used.

Bacteria which were found to lyse both toxic and non-toxic strains of *An. flos-aquae* were isolates number 60, 62, 65, 127 and 145. The first three isolates came from a decomposing *Anabaena* bloom from Beaverhill Lake while the last two are strains of *Cytophaga brunescens* and *Lysobacter enzymogenes* (Table 2). Their identification is discussed in a subsequent section. The experiments conducted did not find a bacterium that would cause lysis in an unshaken culture but not in a shaken one. This was probably due to the fact that rotation of the shaken flasks was not enough to prevent contact or maintain separation between bacteria and algae. This was revealed by microscopic analysis.

3. Toxicity. As already mentioned, the first indication of bacterial effects on toxicity of *An. flos-aquae* came when mature (10- to 20-day) bacteria-free and contaminated cultures of the clone NRC-44-1 were compared (Carmichael, 1972) and the contaminated one was found to be much less toxic. The difference in toxicity noted was at first attributed to bacterial degradation of exogenous toxin for use either as a carbon source, a nitrogen source or a combination of both. Test conditions were at first designed to check for toxicity changes by adding bacteria to a culture which was near its optimum MLD (approximately 60) and testing for loss of toxicity with time. It was

possible, however, that lowered toxicity was due to prevention of toxin production by certain bacteria. To test for this possibility it was necessary to pair bacteria with algae at an earlier stage of the algal growth cycle. This also tested for possible bacterial degradation through reference to the toxicity of the starting culture. Toxicity could be altered from the normal in three ways; lowering, remaining the same, or increasing but not to the level that was expected for the biomass present.

The test conditions that were finally chosen were pairing of bacteria with algae at an OD of approximately 0.2 to 0.3 (five to seven days) and allowing cultures to grow until they attained an OD of 0.6 to 1.0 (nine to eleven days). MLD's from any test were related to a table showing biomass (mg/l) and corresponding MLD's mg/kg within a certain growth range (Table 3). Screening of the bacteria cultures in this way resulted in only two bacterial isolates which showed any effect on toxicity. These were numbers 82 and 85, both isolated from the decomposing *An. flos-aquae* bloom of Beaverhill Lake. Even though algal biomass doubled, toxicity remained unchanged instead of increasing like the controls. Isolate number 82 when added to the algae culture at an OD of 0.2 to 0.3 (MLD = 240) caused no change in toxicity of the culture over a growth period of several days until an OD of 0.6 to 0.8 was attained. It affected the toxicity of a Disney Lake axenic clonal isolate (A-52-2) in the same way. Equal volumes of numbers 82 and 85 (0.5 ml of each) resulted in the same effect as when they were added individually (Table 4). All tests

TABLE 3

Reference table[1] for determining MLD of culture and medium fractions of *An. flos-aquae* NRC-44-1 from its biomass.

Biomass mg/l	OD 750 nm	Culture[2] MLD mg/kg	Medium[3] MLD mg/kg
8*	0.02	960	NT**
19	0.05	640	NT
38	0.10	480	640
58	0.15	320	640
78	0.20	240	480
113	0.30	160	480
152	0.40	80	240
191	0.50	80	160
229	0.60	80	80
268	0.70	80	80
302	0.80	60	60
343	0.90	60	60
381	1.00	60	60

[1]All MLD's were in duplicate. The bioassay has an accuracy of ± ½ MLD step.
[2]MLD of culture as it is grown.
[3]MLD determined after gentle centrifugation (400 *g*) and Millipore filtering.
*Starting culture just inoculated one per cent v/v.
**NT = non-toxic at a dosage of 1,280 mg/kg.

TABLE 4

Effect of bacterial isolates numbers 82 and 85 on toxicity of three axenic clones of *Anabaena flos-aquae*.

Bacteria Isolate Number	Algae Clone	Biomass Algae Culture at Start mg/l	Biomass Algae Culture at Finish mg/ml (4 days)	Viable Bacteria Start	Viable Bacteria Finish	MLD Start	MLD Finish
Control	NRC-44-1	110	240	--------	--------	240	80
82	NRC-44-1	110	205	20×10^5	2×10^7	240	240
85	NRC-44-1	140	330	95×10^4	75×10^5	160	160
Control	A-52-2	50	280	--------	--------	480	80
82	A-52-2	110	210	5×10^5	95×10^5	240	240
85	A-52-2	115	325	2×10^5	80×10^5	160	160
82 + 85	NRC-44-1	50	190	6×10^5	1×10^5	480	320
82	A-113-9	100	250	3×10^5	80×10^5	160	80
85	A-113-9	90	230	4×10^5	90×10^5	160	80

were repeated in triplicate. Values in the table are not averaged but similar results were obtained between runs depending on biomass.

Since the results indicated that toxicity of the culture remained almost stationary when bacteria were added it suggested that they were inhibiting toxin production. Other tests to see how early in the life of the culture toxicity could be controlled were not done, although there was evidence that these bacteria could not completely prevent toxin production if placed with a very young culture. They were most effective in inhibiting toxin production when placed in toxic algal cultures during the log phase of growth. Tests were not done directly on cultures to see whether toxicity of the cell or medium fraction was most affected but it was assumed that if toxicity of the algae culture was being inhibited the amount of toxin in the medium would be correspondingly lower. There was, however, additional evidence to indicate that it was bacterial inhibition of toxin production and not degradation of toxin that was responsible. Toxin extracts in ASM-1-TR were prepared in spectrophotometer cuvettes at a concentration (1 to 5 µg/ml) to give a good extinction value at 229 nm. Equal numbers of bacteria isolates (#82 and #85 were tested separately but not in combination) were placed in the reference and sample cuvettes and 229 nm absorbance followed with time. No change in peak height was observed over time periods of up to 48 hours. Bacterial numbers did not increase or decrease significantly over this time period either. When glucose was added (0.5 mg/ml) to the cuvettes, bacterial numbers increased but the 229 nm peak did

not change. This was interpreted as further evidence that the bacteria did not metabolize the toxin.

The overall implication of bacterial prevention of toxin production is not completely clear. The fact that only two isolates out of 147 were found active indicates either relatively low occurrence in natural blooms and laboratory cultures or more probably that the organic media used to isolate the bacteria did not enrich for these toxin-reducing strains. This would seem to be the case since none affecting toxicity were isolated from NRC-44-1 cultures although they must have been present. The bacteria had no effect under the conditions tested on toxicity of clone A-113-9 from Beaverhill Lake (Table 4). The significant factor was that in the presence of bacteria, the MLD of mature cultures was only 160 to 240 whereas in the bacteria-free condition, the MLD of the same culture at maturity was about 60. It was this finding that made it possible to consistently maintain mass cultures with high yields of toxin for use in studies on toxicology and pharmacology. It did not account for loss of toxicity in laboratory cultures exceeding MLD 240 to 320. This remains to be answered by other data presented in this chapter. It also means that the toxin-affecting bacteria under the conditions tested so far are not primarily responsible for toxic and non-toxic blooms.

B. Identification of Bacteria Affecting Growth and Toxicity of *Anabaena flos-aquae*

Of the five bacterial isolates that were found to lyse *Anabaena flos-aquae* three needed identification. These three have been identified as belonging to the family Enterobacteriaceae. This was based on their being Gram-negative motile rods, ability to ferment glucose with the formation of acid and gas (Board and Holdings media, Table 5 and Appendix 3) and their ability to reduce nitrates (Penassay plus nitrates or nitrites, Table 5 and Appendix 3). These are the prime characters of this family as listed by Edwards and Ewing (1972) and in Bergey's Manual of Determinative Bacteriology (Breed, *et al.*, 1957). Enterobacters are frequently found in the alimentary, respiratory and urinary tracts of vertebrates but some are free-living. The three isolates from this work do not grow well at elevated temperatures (37^{o}C) which indicates that they are free-living strains adapted to natural water temperatures. Further biochemical tests placing these isolates within the family were from the results of the API (Analytab Products Inc.) 20 tab test, plus the DIFCO table for differentiation of Enterobacteriaceae. Results from these tests indicated that the three isolates are in the tribe Klebsiellae and most probably are all in the genus *Pectobacterium* (Table 5). The characteristics from Table 5 that separate these three isolates into the Pectobacteria include the negative tests for both fermentation of sorbitol and gas production from inositol. A positive

TABLE 5

Biochemical tests and results used in identification of bacteria isolates number 60, 62, 65, 82 and 85. ("+" = positive color reaction or growth; "-" = no color reaction or growth)

Isolate	60	62	65	82	85
Board and Holdings (acid + gas produced)	+	+	+	+	-
Penassay broth	+	+	+	+	-
Penassay + Nitrate	+	+	+	+	-
Penassay + Nitrite	+	+	+	+	-
Skim milk (Clear)	+	+	+	slight	-
β-galactosidase (ONPG)	+	+	+		
Arginine dehydrolase (ADH)	+	+	+		
Lysine decarboxylase (LDC)	-	+	+		
Ornithine decarboxylase (ODC)	-	-	-		
Citrate	-	+	+		
Hydrogen sulfide	-	-	-		
Urease	-	+	-		
Tryptophane deaminase (TDA)	-	-	-		
Indole	+	+	+		
Acetoin	+	+	+		
Gelatin	+	+	+		
Glucose	+	+	+		
Mannitol	+	+	+		
Inositol	-	-	-		
Sorbitol	-	-	+		
Rhamnose	-	-	-		
Saccharose	+	+	+		
Melibiose	-	-	-		
Amygdaline	+	+	+		
Arabinose	+	+	+		
Glucose nitrates	+	+	+		

test for liquification of gelatin and carboxymethylcellulose is also characteristic of this genus. They grow well on skim milk media which is also characteristic of *Cytophaga*. These results plus the fact that Pectobacteria grow poorly or not at all at 37°C (optimum temperature is 25°C) (Edwards and Ewing, 1972) would favor placement of these lytic isolates in this genus. This family and genus of bacteria has not been reported lytic to blue-green algae but because of the characteristics of this group it is not too surprising that they are lytic under the proper circumstances. Under what conditions they could become abundant enough in a bloom to effect its growth has not been studied. It can be considered that due to their facultative ability for anaerobic growth they could affect algal lysis under conditions of heavy algal concentration and low oxygen tension. This would be in contrast to *Cytophaga* sp. which lyse algae but are obligate aerobes. It was also noticed that these three cultures are contaminated with what appears to be a *Bdellovibrio* type of bacteria which are much smaller rods (0.5 to 1.0 μ *vs* 5.0 to 10.0 μ) and have been reported to be parasites on other bacteria (F. D. Cook, personal communication). It is difficult to remove these smaller rods from their association with the Pectobacteria but to be sure of their lytic role it should be done. The other lytic strains 127 and 145 which are *Cytophaga brunescens* and *Lysobacter enzymogenes* respectively are from the F. D. Cook culture collection. These would be expected to have lytic properties but it is interesting that none of the other 18 strains tested from this collection showed any lytic potential.

It implies that the lytic enzymes are quite specific to strains within even a species.

Isolates 82 and 85 which affect toxicity of certain *An. flos-aquae* toxic clones have not been identified to the genus level at this time. Number 82 has several of the characteristics of the lytic strains such as fermenting glucose to acid and gas and reduction of nitrates. It does not, however, lyse *Anabaena* nor does it liquify gelatin. It would seem to belong to the tribe Klebsielleae but the choice of genus has not been made. Number 85 seems not even to be in this same family as it does not ferment glucose to acid and gas nor reduce nitrates. It should be noted, however, that 85 has become contaminated with at least two other rods since it was tested on the algal cultures. More work will have to be done with both these isolates before their identity is known.

C. Colony Toxicity and its Relation to Toxic Blooms

As mentioned earlier, Gorham, *et al.* (1964) found that unialgal colony isolates of *Anabaena flos-aquae* from the same bloom varied in toxicity. These colony isolates also varied greatly in toxicity from subculture to subculture. In addition, a number of single-filament isolates (clones) from two colony isolates, NRC-44 and NRC-36, varied in toxicity (Gorham, personal communication). This same pattern was found in the colony isolates made from the Disney Lake samples collected on June 25, 1972. Fifteen colony isolates from this lake had MLD's ranging from 160 to 960 when

bioassayed four months after isolation. (It took this length of time to grow the colonies, check for protozoa or other algal contaminants, recheck the cultures if necessary and then grow sufficient quantities under the same physical conditions for toxicity comparisons.) Periodic checking of the toxicity of these colony isolates over the subsequent twenty-month period showed a trend in all but two (A-48-3 and A-76) toward the non-toxic condition (Table 6).

The results in Table 6 show that colony isolates subcultured over a period of time lose toxicity. Since the presence of bacteria does not appear to eliminate toxicity, only reduce it so that MLD's are higher by about 240 to 320 mg/kg, there are two possible explanations for this trend to the non-toxic condition. First, it could be a genetic change so that the filaments produce less and less toxin even though physical conditions remain the same. Second, the colony isolate could originally have been composed of toxic and non-toxic filaments which, upon repeated subculturing resulted in the non-toxic component predominating. To test these possibilities required some extensive manipulations of individual colony isolates. That genetic loss of toxicity was occurring seemed unlikely since NRC-44-1 was producing such a constant yield of toxin over time after it was obtained in the axenic condition. This left the second possibility. To examine this possibility, numerous single-filament isolations and two multifilament isolations from the colony cultures were done and their toxicities monitored with time. Since the full extent of the trend to the non-toxic condition was not realized until some colony

TABLE 6

Colony isolates from Disney Lake and their toxicities over a 20-month period.

Colony isolate numbers Disney Lake, Alberta *An. flos-aquae*	MLD mg/kg (OD 0.4 to 0.8)			
	Months after isolation			
	4	10	15	20
A-47-1*	240	640	---	NT†
A-48-2*	160	320	---	NT
A-48-3*	160	160	---	160
A-49-1*	960	960	---	NT
A-52	640	---	960	NT
A-53	320	---	---	NT
A-61	640	960	---	NT
A-68	240	1,280	---	NT
A-70	120	960	960	NT
A-71	240	---	NT	NT
A-73	160	---	---	NT
A-74	240	---	---	NT
A-76	320	---	---	320
A-77	320	---	---	NT
A-78	240	---	---	NT

*Numbers after the original isolation number indicate that the original colony had multifilament subisolates made from it and these are the survivors.

†NT = non-toxic at a dosage of 1,280 mg/kg

isolates had become non-toxic, only a few colony isolates remained in the toxic condition to serve as parental material. Axenic toxic clones of A-52, A-70 and A-71 (Carmichael and Gorham, 1974) were obtained approximately 12 months (summer of 1973) after the original colony isolations. These clones have maintained a constant level of toxin production (MLD = 60) with time (Table 7) like NRC-44-1. Other isolates from A-52 which were not single filaments have shown loss in toxicity with time (Table 7).

Other colony isolates were then checked for this same trend, choosing those colony isolates that still had some toxicity (A-48-3 and A-76) and comparing them with colony isolates (A-48-2 and A-73) that had lost their toxicity. Toxicity tests of clonal isolates from these colonies are also given in Table 7. All MLD's are from cultures having an OD 750 of 0.4 to 0.8, unless otherwise noted. Allowing six mice per test and including the many replicates and extras not given in Table 7 there were about 1,000 mice used for this portion of the research.

From the results in Tables 6 and 7 the following conclusions can be drawn:

1. Colony isolates from a toxic bloom show variable toxicity when cultured under the same physical conditions.
2. Subcultures from colony isolates of a toxic bloom show a distinct trend toward the non-toxic condition (Table 6).
3. Multifilament isolates (A-52-4(m); A-52-6(m)) from a colony culture show an initial MLD that is higher than accountable by bacterial presence

TABLE 7

Clonal and multifilament isolates from Disney Lake colony isolates and their toxicities with time. Except where noted these isolates are non-axenic.

Isolate Number**	MLD mg/kg		
	Months after isolation		
	1	3	4
A-52-2* (axenic)	60	60	60
A-52-3* (axenic)	NT***	---	NT
A-52-4{m}*	640	NT	NT
A-52-6{m}*	480	NT	NT
A-52-4-a†	NT	---	NT
A-52-4-b	---	---	NT
A-52-4-c	---	---	NT
A-52-4-d	---	---	NT
A-52-4-e	---	---	NT
A-52-4-f	---	---	NT
A-52-5*	160	240	---
A-52-5-a†	---	---	240
A-52-5-b	---	---	240
A-52-5-c	---	---	160
A-52-5-d	---	---	120
A-70-10* (axenic)	60	60	60
A-70-11* (axenic)	NT	---	NT
A-71-2* (axenic)	60	60	60

Table 7 (cont.)

Isolate Number	MLD mg/kg		
	Months after isolation		
	1	3	4
A-48-3-a†	240	---	---
A-48-3-b	320	---	---
A-48-3-c	240	---	---
A-48-3-d	80	---	---
A-76-1†	240	---	---
A-76-2	320	---	---
A-76-3	160	---	---
A-48-2-a†	NT	---	---
A-48-2-b	NT	---	---
A-48-2-c	NT	---	---
A-48-2-d	NT	NT	---
A-48-2-d-1	NT	---	---
A-48-2-d-2	NT	---	---
A-48-2-d-3	NT	---	---
A-48-2-d-4	NT	---	---
A-73-3	NT	---	NT
A-73-3-a	NT	---	---

*Isolated between the four to ten month period of Table 5.
†These isolations were made during the period of October 1973 to January 1974.
**See page 19 for isolate coding procedure.
***NT = non-toxic at a dosage of 1,280 mg/kg.

and if tested over time the culture proceeds to the non-toxic condition.

4. Toxic clones which are axenic (A-52-2, A-70-10 and A-71-2) have stable optimum MLD's of approximately 60 (for an OD of 0.5 to 0.8).

5. Unialgal single filament isolates (clones) from a colony culture (*e.g.*, A-52) are toxic and non-toxic. If toxic (A-52-5-a to d) their toxicity is within the range accounted for by bacterial presence. It also appears that the initial bacterial flora can be qualitatively different allowing for the toxicities of a unialgal clone (A-48-3-d) to approach that of an axenic clone.

To further verify the observations that colonies of *An. flos-aquae* from a toxic bloom are not as homogeneous as formerly supposed but frequently consist of mixtures of toxic and non-toxic filaments, mixes of axenic toxic and non-toxic clones in known proportions were made and tested for toxicity. Within the limits imposed by the precision of the bioassay, these mixes were expected to have an MLD in proportion to the ratio of toxic and non-toxic filaments present. These results (Table 8) for mixes of axenic NRC-44-1 (toxic), with A-52-3 (non-toxic) and A-70-11 (non-toxic) indicate, as expected, that mixtures of toxic and non-toxic filaments give MLD's in proportion to their respective ratios.

The MLD's reported for 14 colony isolates from Burton Lake (Gorham, *et al.*, 1964) can also be interpreted on the same basis. They found eight colony isolates to be toxic and six non-toxic. The toxic strains had low toxicities (MLD's 640 to 1,280) which would indicate that a large proportion of the filaments in the cultures were non-toxic at the time of bioassay. These studies help to explain a good deal

TABLE 8

Mixtures of axenic toxic and non-toxic clones of *Anabaena flos-aquae* and their toxicity.

Clonal mix	Filament ratio at time of bioassay	OD	MLD
NRC-44-1: A-52-3 NT*	9:1	0.54	60
NRC-44-1: A-52-3 NT	4:1	0.60	60
NRC-44-1: A-70-11 NT	1:1	0.70	120
NRC-44-1: A-70-11 NT	1:2	0.70	240

*NT = non-toxic at a dosage of 1,280 mg/kg.

of the variable toxicity of laboratory-grown colony isolates and clones derived from them but the results should only be applied to the interpretation of the field situation with caution until more direct observations have been made. It would appear that the variable toxicity of a bloom is primarily determined by the relative proportions of toxic to non-toxic colonies and of toxic to non-toxic filaments within the colonies. The role of lytic and toxin-degrading bacteria which may be present in blooms is probably secondary. This implies that a toxic component exists in many if not all *Anabaena* blooms which, in most, but not all instances, remains below the threshold that is harmful to animals. The two important questions that remain unanswered include; what factor or factors select for predominant growth of toxic or non-toxic filaments with time in a bloom, and the factor or factors that select for non-toxic filaments to become dominant in time with mixed cultures in the laboratory.

D. Toxicology of *Anabaena flos-aquae*

1. General. Symptoms of poisonings by *An. flos-aquae* have been mainly from field observations. Descriptions tend to vary somewhat among observers, however there are certain effects which have been reported fairly consistently. Poisonings of mammals, which include mainly dogs, cows, horses and, in the laboratory, mice and rats, generally involve twitching, staggers, convulsions and gasping breath, with death occurring in a few minutes for smaller animals and in one to three hours for larger animals, depending on dose. In

birds, which include mainly geese, ducks, gulls and pigeons, the usual symptoms are extension of legs, contracture, flopping and extension of the neck back over the wings. Certain of these same symptoms have been observed in pigeons dosed in the laboratory. In one case these symptoms have been described by the clinical term of opisthotonos (Fitch, Bishop and Boyd, 1934). Symptoms of poisonings in selected cases where *Anabaena* has formed at least part of the bloom are summarized in Table 9. Information on the amounts of toxic *An. flos-aquae* bloom that were ingested to cause sickness or death is, unfortunately, less abundant than observations on the symptoms preceding death.

Animals selected for toxicology studies were mainly those whose loss was of economic concern. This included calves, ducks and goldfish, representing livestock, waterfowl and fish, respectively. Fish losses have not been directly attributed to *Anabaena* toxin although losses caused by anoxia created by decomposing blooms are well known. It was thought important to check the response of fish to certain levels of *Anabaena* toxin because *Aphanizomenon flos-aquae* toxin has been reported to kill fish in a natural bloom (Sawyer, Gentile and Sasner, 1968). Other animals were chosen for toxicological tests based on their value in determining basic MLD levels (mice and rats).

As mentioned earlier, material used in the assays was either lyophilized cells or toxin extract. The lyophilized cell material used in most cases had an MLD of 60 (IP mice). The toxin extracts

TABLE 9

Symptoms of algal poisonings from reports in which *Anabaena flos-aquae* was mentioned as composing part or all of the bloom.

Case	Animal	Dosage	Symptoms	Bloom Composition	Reference
Lake Lac qui Parle, Milan, Minn. Oct., 1933	Rabbits Pigeons	Oral, No amount cited	Rabbits: opisthotonos Pigeons: opisthotonos Head drawn back; Death 10 to 15 minutes	*Microcystis*, *Anabaena*, *Aphanizomenon*	Fitch, Bishop and Boyd, (1934)
Sturgeon Lake, Fenelon Falls, Ontario July, 1949	Cow Rabbits Pigeons	Oral, Cow ≈ 273 g Rabbit ≈ 300 mg IP, Pigeon: 5 ml filtrate	Cow: twitching, staggers; death 25 minutes Rabbit: contracture forelimbs, gasping, deep respiration; death 10 min. Pigeon: stiffening of neck & legs, legs outstretched, neck rigid, head drawn back	*Microcystis*, *Anabaena*, *Nostoc*, *Lyngbya*	Stewart, Barnum and Henderson, (1950)
Storm Lake, Buena Vista County, Iowa Oct., 1952	Birds, mainly Franklin's Gulls	Unknown	Head flopping, legs extended rigidly out behind	*Anabaena*	Rose, (1953); and Des Moines Register, Iowa, (Nov. 23, 1952)

Table 9 (cont.)

Case	Animal	Dosage	Symptoms	Bloom Composition	Reference
Echo Lake, Near Regina, Saskatchewan June, 1959	Geese	Unknown	Death preceded by craning of neck	*Anabaena, Microcystis*	Dillenberg and Dehnel, (1959)
Lake Reno, Near Glenwood, Minnesota Sept., 1955	Mice Ducks	Oral, "Small amount of natural bloom"	Mice: stretching, convulsions, sometimes foamy tears; death 2 to 3 minutes Ducks: fall forward, feet back, head and neck drawn back over shoulder in "S"-shaped curve; death in 20 to 22 minutes. Nictitating membrane not paralysed.	*Anabaena Lemmermanni (=flos-aquae)*	Olson, (1964)

used were those obtained from 40 g of MLD 60 cell material (Figure 3). The amounts and MLD's of the three extracts were: (A) 143.5 mg of MLD = 0.3; (B) 63.5 mg of MLD = 1.0; (C) 30.9 mg of MLD = 2.5. The extract used for most of the studies was (A). For assays the cell material was made up to a known concentration with distilled water and the extract was made up in either distilled water or appropriate animal saline (Appendix 4).

2. Rats. Initial experiments on general toxicological responses were done with rats because their symptoms could be compared to mice and they were also readily available. MLD, IP and oral, was determined using duplicate animals. The dose levels used were the same as those used for mice. The dose level was determined in the following manner:

1. An aqueous suspension containing 32 mg/ml of lyophilized cells was prepared.
2. A 300 g rat would then receive three ml to test a response for a 320 mg/kg dose level.
3. If both rats died the dose would be halved and response tested for 160 mg/kg.

In this way the MLD for rats was found to be 60 (IP) and 25 times this, or 1,500, orally. Rats were given oral doses of the cell suspension by means of a metal tube fitted to a syringe. The tube was inserted down the esophagus and the cell suspension injected into the stomach. Survival times were 10 to 12 minutes IP and 12 to 15 minutes orally.

Symptoms of death were similar to those of mice except that the survival times were about twice as long. These included a latent period of two to four minutes followed by a period of little active movement with tremors or stretching of hind limbs. Then came periods of gasping, twitchings and convulsions. It appeared that death was due to respiratory arrest. Autopsy would reveal, as in the mice, the heart still beating with arrhythmia of the atria and contraction mostly confined to the ventricles. Internal organs were normal looking but blood seemed to be concentrated in the area of the visceral organs leaving the peripheral regions pale.

3. Calves. Since calves and livestock represent a considerable economic loss when killed by *Anabaena* toxin, it was important to get some idea of the oral MLD and the accompanying symptoms. Since experiments on rats had been done which showed the toxin to be neuromuscular and that artificial respiration could maintain normal heart rate and blood pressure (see section under Pharmacology, Rats) it was decided to check the same response with calves to see if it was possible with artificial respiration to maintain the animal and permit detoxification and return of muscle control to occur. The oral dose necessary for death had to be guessed at on the basis of data for the rat. If it was the same amount on a weight basis as for the rat, *i.e.* 1,500 mg/kg, the oral MLD for a 60 kg calf would be about 90 g.

Two weaned calves, whose rumen flora would be representative of older animals on a grain and hay diet, were used in the tests with

the procedures and results as follows:

Calf number one was a male Black Angus weighing 73 kg (165 pounds) ID number 7406. He was about six weeks old and had been weaned at four weeks. The calf was starved overnight and dosed with 160 g of lyophilized NRC-44-1 (MLD = 80), according to the protocol and with the results described in Table 10.

Results from the first calf experiment indicated that the algae dose was in excess of an MLD. Due to short survival time it was estimated that it was probably about twice the MLD. This would make calves about twice as sensitive by the oral route as rats. The results also showed that an anticholinesterase had no apparent effect on neuromuscular response. Finally, over the time period of the experiment, detoxification by the animal had occurred very little, if at all. To better estimate the oral MLD for calves and to check the possibility that at, or just a bit more than, an MLD, artificial respiration would permit the animal to regain muscle control, a second calf was tested on May 2. This second calf was a male (ID number 7411), five weeks and four days old, weighing 42.5 kg. He had been weaned to a grain and hay diet at four weeks of age. For this experiment it was decided to give the animal what were considered to be sublethal oral doses of the aqueous cell suspension at different time intervals. Since it was estimated that the calf oral MLD was ten times the mouse IP MLD/kg, the dose levels chosen were 2.5, 2.5, 1.25 and 2.5 times. These dosages were to be given at about ten-minute intervals until the calf collapsed with respiratory arrest. This should then be

TABLE 10

Experimental procedure and results for oral dosing of a 73 kg calf (ID number 7406) with a suspension of lyophilized *Anabaena flos-aquae* NRC-44-1 (MLD IP mouse = 80).

Time	Event or Observation
0 min (1015)	Cannulation of jugular vein and insertion of a two-way valve and syringe with saline for taking blood samples to monitor gases and pH. This was to enable monitoring and regulation of artificial respiration so that hyper- or hypoventilation did not occur.
18 min (1033)	Dosed, by a rubber tube inserted into the reticulo-rumen, with 160 g of lyophilized cells. Material was suspended in 700 ml water and washed into reticulorumen by 300 ml water. This material was injected through the stomach tube with a 100 ml syringe. First blood sample was taken to get normal level of blood gases and pH. The first sample was wasted but control levels of blood gases were estimated to be about PO_2 20 mm Hg; PCO_2 40 mm Hg; pH 7.2.
22 min (1037)	Staggers, convulsions. Animal collapsed. Breathing was abdominal. Intubation of trachea for artificial respiration was started.
28 min (1043)	Heart rate approximately two times faster with respirator.
31 min (1046)	Second blood sample: PO_2 19.5; PCO_2 72; pH 6.91.
42 min (1057)	Third blood sample: PO_2 25.5; PCO_2 50; pH 7.03.
47 min (1102)	Heart rate 200/min., some arrhythmia.

Table 10 (cont.)

Time	Event or Observation
50 min (1105)	Fourth blood sample: PO_2 25; PCO_2 35; pH 7.13.
75 min (1130)	Fifth blood sample: PO_2 25; PCO_2 37; pH 7.18.
79 min (1134)	1 ml (10 mg) of an anticholinesterase, edrophonium chloride, was injected IV (jugular) with no effect.
110 min (1205)	Respirator changed to get a more even respiration and blood gases.
125 min (1220)	Sixth blood sample: PO_2 52.5; PCO_2 47; pH 7.23
130 min (1225)	Electrocardiogram (ECG) and blood pressure were measured using Hewlett Packard 4-channel physiograph. Femoral artery used for pressure measurement. Poor electrode contact appeared responsible for erratic PQRST complex but ECG appeared essentially normal. Blood pressure was averaging about 100 mm Hg.
190 min (1325)	Seventh blood sample: PO_2 42; PCO_2 64; pH 7.13.
233 min (1408)	Decided to terminate experiment because of suspected high dose of algae given to the animal and no indication of any return of muscle coordination. Respirator turned off.
239 min (1414)	P-wave of ECG gone. (See Pharmacology section, Rats).
260 min (1435)	Autopsy done to check condition of organs and to trace how far algae filaments had been distributed through digestive system. Autopsy showed all organs appearing normal. Reticulorumen contents were green-tinged. Microscopic examination showed filaments present in all stomachs; reticulorumen, omassum and abomassum.

very close to the MLD of the toxin. At this level of toxicity, artificial respiration might allow detoxification to occur. The experimental procedure and results are outlined in Table 11.

The results of the calf experiments indicate the following:

1. The oral MLD for calves is between six and eight times the MLD (IP mouse)/kg. This was concluded on the basis of the second calf which collapsed with respiratory arrest after an 8.75 times dosage. This dosage administered in sequential sublethal amounts resulted in tachyphylaxis so that it was likely that the acute MLD had been exceeded. This MLD is within the range that animals in the field could be expected to consume in one drinking (360 to 480 mg/kg at a bloom concentration of about 10 mg/ml which would be equivalent to 2.5 liters for a 60 kg calf). This is compared with nine liters of waterbloom had the oral MLD been the same as a rat, *i.e.* 1,500 mg/kg.
2. Artificial respiration could restore normal heart rate, blood pressure, blood gases and pH after respiratory collapse due to the toxin. Sufficient detoxification did not occur during approximately 30 hours of artificial respiration to allow return of peripheral muscle control. Since artificial respiration could maintain certain vital body functions it was taken as more evidence for the toxin having a neuromuscular blocking mechanism of action.
3. The anticholinesterases edrophonium and neostigmine which are effective in preventing curare or curare-like competitive post-synaptic block were ineffective against the toxin. While some muscle twitches were seen after administration of these drugs it was not

TABLE 11

Experimental procedure and results for sequential sublethal oral dosing of a 42.5 kg calf (ID number 7411) with a suspension of lyophilized *Anabaena flos-aquae* NRC-44-1 (MLD IP mouse = 60).

Time	Event or Observation
0 min (1043)	Jugular vein cannulated for blood samples.
7 min (1050)	First blood sample: PO_2 31.8 mm Hg; PCO_2 < 10 mm Hg; pH (?) (not enough sample).
17 min (1100)	Dose #1: 6.3 g (2.5 X MLD/kg IP mouse). Given orally by stomach tube.
32 min (1115)	Jugular cannula plugged, removed.
44 min (1127)	Second blood sample taken directly by syringe into jugular vein: PO_2 31.5; PCO_2 30.5; pH 7.43.
47 min (1130)	Dose #2: 6.3 g (Total = 5 X MLD/kg IP mouse).
50 min (1133)	Muscle fasiculations (tremors) in shoulder.
57 min (1140)	Fasiculation in limbs.
60 min (1143)	Animal prostrate but still breathing.
65 min (1148)	Some paralysis (ptosis) of upper eyelid.
70 min (1153)	Third blood sample: PO_2 27; PCO_2 32.5; pH 7.38.
75 min (1158)	Dose #3: 3.15 g (Total 6.25 X MLD/kg IP mouse). Respiration 14/min.
90 min (1213)	Fourth blood sample: PO_2 27; PCO_2 31; pH 7.40.
107 min (1230)	Dose #4: 6.3 g (Total 8.75 X MLD/kg IP mouse).
112 min (1235)	Heart rate 78/min., respiration 14/min. Absence of definite response to the last two doses indicated tachyphylaxis (resistance to increased sublethal doses of the toxin).

Table 11 (cont.)

Time	Event or Observation
122 min (1245)	Ear, eye and pain reflex are still present (manual stimulation). Fifth blood sample: PO_2 31.2; PCO_2 30.5; pH 7.38.
152 min (1315)	Sixth blood sample: PO_2 33.5; PCO_2 28.5; pH 7.35.
227 min (1430)	Animal made attempts to rise over period of next 60 minutes.
290 min (1538)	Irregular breathing with convulsions.
292 min (1540)	Respiratory collapse, animal placed on respirator.
301 min (1551)	IV injection of 10 mg edrophonium chloride with no effect. Seventh blood sample: PO_2 134.5; PCO_2 49.5; pH 6.92. Hyperventilation. Respirator rate decreased.
325 min (1615)	Rectal temperature 98^{o}F.
370 min (1700)	Heart rate 60/min.
387 min (1717)	IV saline drip started to prevent dehydration of the animal.
460 min (1830)	Heart rate 90/min and steady.
484 min (1854)	10 mg edrophonium chloride (IV) caused some convulsive breathing.
487 min (1857)	Respirator removed and breathing continued for a short time but was not steady.
490 min (1900)	Respirator restored. Heart irregular and 60/min.
500 min (1910)	Animal began urination which continued intermittently until saline drip was stopped.
502 min (1912)	Five per cent dextrose saline drip started (IV).

Table 11 (cont.)

Time	Event or Observation
508 min (1918)	0.25 mg Neostigmine (IV) (an anticholinesterase higher in activity and longer acting than edrophonium). Over approximately the next two-hour period there was occasional convulsive breathing. ECG was continually monitored from this time on. Heart wave pattern was normal with average heart rate under artificial respiration at 90/min.
655 min (2145)	Animal secured to operating table and left for next three hours and 45 minutes. Saline drip stopped (approximately two liters used).
880 min (0130)	No ear or eye reflex. Some pain reflex. Animal shows general prolapse (ptosis) of rectal muscles.
895 min (0145)	Respirator removed with some shallow abdominal breathing evident for about three minutes.
902 min (0152)	Respirator restored. This on-off procedure was repeated over a 20-minute period until the animal began normal respiration.
925 min (0215)	Animal regained normal respiration with a rate of 33/min and a heart rate of about 100/min. Intubator was removed. Animal maintained normal respiration, heart rate and body temperature for next several hours.
1320 min (0850)	Eighth blood sample: PO_2 22.8; PCO_2 39.5; pH 7.35. Animal was put under heat lamp.
1345 min (0915)	Animal was put back on glucose-saline drip (IV).
1392 min (1002)	Ninth blood sample: PO_2 25; PCO_2 41; pH 7.28.

Table 11 (cont.)

	Event or Observation
1500 min (1150)	Respiratory collapse. Animal was intubated and placed on respirator.
1565 min (1245)	IV drip was stopped.
1700 min (1500)	No reflexes from the animal. Experiment ended by shutting off respirator. Autopsy showed all organs apparently normal. Microscopic examination of contents of recitulorumen, omassum and abomassum showed small amounts of fragmented algal filaments in all three. Filaments were longer and more common in the reticulorumen.

permanent and indicated only temporary potentiation of acetylcholine present in the nerve muscle junction.

4. Autopsy showed that algae filaments had been distributed throughout the digestive tract of the calves. The various compartments of the ruminant system and the papillae of the digestive system were indicative of animals who are on a hay and grain diet (Church, 1969). Because of this it was felt that the oral MLD values and symptoms of the toxin could be related to dairy or beef livestock who are poisoned by toxic blooms in the field.

An indication of the experimental arrangements for the calf experiments is given in Plate 4.

4. Ducks. Male mallard ducks were used to obtain symptoms and IP and oral MLD's for a typical waterfowl species. The ducks were approximately two years old and weighed about one kilogram. They were given water but no food for 24 hours prior to the test. Lyophilized cells (MLD, IP mouse = 60) were made up to concentration levels in the range similar to those of mice and rats. Animals were given oral dosages of the cell suspensions through a 0.317 cm inside diameter, 0.635 cm outside diameter, 23-cm piece of Tygon tubing. The tip of the tubing was flame-smoothed to minimize injury to the esophagus. The dosages, symptoms and death times for both IP and oral route are given in Table 12.

The results of Table 12 reveal several points about the toxicology of *An. flos-aquae* in ducks and with inference to other birds

PLATE 4. General facilities for the calf experiment showing artificial respiration and ECG monitoring. (Surgical Medical Research Institute).

TABLE 12

Results of oral and IP doses on male mallard ducks with a suspension of lyophilized *Anabaena flos-aquae* NRC-44-1 (MLD IP mouse = 60).

Time, min	Dose; Route	Animal Weight, g	Event or Observation
0	35 mg/kg; IP	980	Injection just below sternum.
6			Legs stiff and projected behind body, neck straight out.
9			Some loss of coordination.
11			Some gasping respiration.
8			Neck control regained.
22			Trying to regain standing position.
35			Legs still outstretched but normal breathing and neck control.
160			Recovery almost complete, normal posture.
0	53 mg/kg; IP	760	Injection just below sternum
2			Legs stiff and projected behind body.
3			Rigid body contracture, neck stiff and thrown back over body. Gasping respiration.
8			Respiration intermittent and gasping. Heartbeat strong and steady.

Table 12 (cont.)

Time, min	Dose; Route	Animal Weight, g	Event or Observation
11			Convulsive wing beat. Dead. Nictitating membrane not paralyzed. Autopsy showed liver, intestines, pancreas, *etc.* to be normal in appearance. There was no hemorrhage or inflamation of tissue.
0	155 mg/kg; oral	970	Dosed. Cell suspension washed through tube with 5 ml of water.
4			Feet extended out behind.
7			Irregular breathing.
12			Head normal, feet still extended.
46			Almost normal feet and neck movements.
59			Returned to cage, normal.
0	250 mg/kg; oral	890	Dosed. Cell suspension washed through tube with 5 ml of water.
4			Feet extended.
6			Feet normal, flapping wings.
11			Head thrown back over shoulder with slight wobbling of head from side to side.
15			Head back, legs extended, gasping respiration.
22			Dead. Heart beat still strong but irregular.

Table 12 (cont.)

Time, min	Dose; Route	Animal Weight, g	Event or Observation
0	300 mg/kg; oral	1,000	Dosed. Washed through tube with 5 ml of water.
3			Legs extended.
7			Gasping respiration, neck not contracted back over shoulder.
24			Head flopped over on side.
32			Leg contracture decreasing.
62			Normal.
0	385 mg/kg; oral	905	Dosed. Washed through tube with 5 ml of water.
2			Legs extended.
3			Head wobble and back slightly over shoulder.
4			Neck contracted back.
14			Dead. Convulsions, wings flapping and gasping respiration.
0	370 mg/kg; oral	940	First of four doses given. Washed through tube with 5 ml of water.
9			Legs extended.
27	160 mg/kg; oral Total: 530 mg/kg		Second dose given.
37			Legs extended, breathing normal.

Table 12 (cont.)

Time, min	Dose; Route	Animal Weight g	Event or Observation
40	210 mg/kg; oral Total: 740 mg/kg		Third dose given. No change.
57	530 mg/kg; oral Total: 1,270 mg/kg		Fourth dose given.
65			Unable to move, legs extended but not rigid contracture. Head flopped to side, not contracted back.
75			Breathing shallow but steady.
105			No pain reflexes. Heartbeat steady.
110			Heartbeat slowing.
115			Dead. Heart still beating. Slow death time and high total dosage indicate tachyphylaxis. Autopsied; algal filaments along entire intestinal tract.

and waterfowl. The extension of the legs out and behind the body followed by contracture of the neck back over the shoulder in an "S"-shaped curve (Table 9 and Plate 5) is similar to that reported by others. It also resembles opisthotonus which was first described in chicks by Buttle and Zaimis (1949) when decamethonium was injected (Plate 9). Decamethonium is a synthetic compound that is used to demonstrate postsynaptic depolarization of the nerve-muscle junction. The IP MLD for mallard ducks was estimated to be about 50 mg/kg. The oral MLD based on these tests is estimated to be about seven times the IP MLD for ducks or six times the IP MLD = 350 mg/kg for mice. This was concluded on the basis that the duck given 385 mg/kg was nearer to the MLD than the duck that died at 250 mg/kg. On the basis of 10 mg/ml of biomass for a heavy waterbloom, 35 ml would be adequate for death to occur in a 1 kg duck. Tachyphylaxis which is characteristic of depolarizing drugs, was observed in the dosing of one of the ducks. Autopsy of the ducks given oral dose indicated that the algal suspension was distributed throughout the entire digestive system. In ducks this would be described as follows: An esophagus long and large in diameter with a crop not differentiated as in other birds such as chickens or pigeons. This leads into the anterior glandular stomach (proventriculus) and a posterior muscular stomach (ventriculus or gizzard). Ducks also have a pyloric chamber between the gizzard and intestine. This is followed by the small and large intestine (Farner, 1960).

5. Goldfish. While fish kills have not been reported which are directly linked with toxic waterblooms of *An. flos-aquae* there was

PLATE 5. Male mallard duck given an oral MLD (mg/kg) of lyophilized cells of *Anabaena flos-aquae* NRC-44-1. Death occurred in 15 minutes. Note opisthotonus.

no reason to suspect that fish would be unresponsive to the toxin. Duplicate goldfish weighing about 20 to 30 g were used for each dosage tested. The effects of oral doses of cell suspension (MLD IP mouse = 60) and IP doses of both cell suspension and extract (fraction B, MLD IP mouse = 1.0) were examined. For oral doses the syringe with a metal tube used for the rats was employed. The fish were kept in aerated dechlorinated water during the test period. The results of the tests are summarized in Table 13.

These results indicate several important points about the potential for fish poisoning by *An. flos-aquae*. The oral and IP MLD for the cell suspension was the same (160) indicating equal absorption of the toxin by both routes. By the intraperitoneal route, goldfish are about one-half as sensitive as rats, mice and ducks. By the oral route, however, their sensitivity is much greater than rats, mice, ducks and calves. The IP MLD for the extract also indicates that they are about one-half as sensitive as rats and mice. The general symptoms of death in fish, such as they can be deduced from visual observation, indicate a type of muscle contracture which causes difficulty but not complete loss of the ability to maintain equilibrium in the water. The cause of death appears to be inability to operate the respiratory pump, *i.e.* the movement of the buccal and bronchial cavities for intake of water (Prosser, 1973) (Plate 6). In some cases, particularly with doses in excess of the MLD, the mouth contracts in the open position.

An oral MLD implies actual ingestion of the algae for death to occur. With a toxic waterbloom of *An. flos-aquae* there is also the

TABLE 13

Results of oral and IP doses on goldfish of *Anabaena floe-aquae* NRC-44-1.

Time, min	Dose; Route	Animal Weight, g	Event or Observation
0	320 mg/kg; IP - cells	28	Dosed.
2			Inactive, rigid, can not maintain equilibrium.
6			Only slight movement of gills; floats on side or back. Mouth contracted open.
11			Dead.
0	320 mg/kg; IP - cells	30	Dosed.
13			Dead. Symptoms same as above.
0	160 mg/kg; IP - cells	25	Dosed.
13			Dead.
0	80 mg/kg; IP - cells	20	Dosed.
20			Slight difficulty maintaining equilibrium.
50			Normal, only slight effects.
0	80 mg/kg; IP - cells	26	Dosed.
50			Normal.

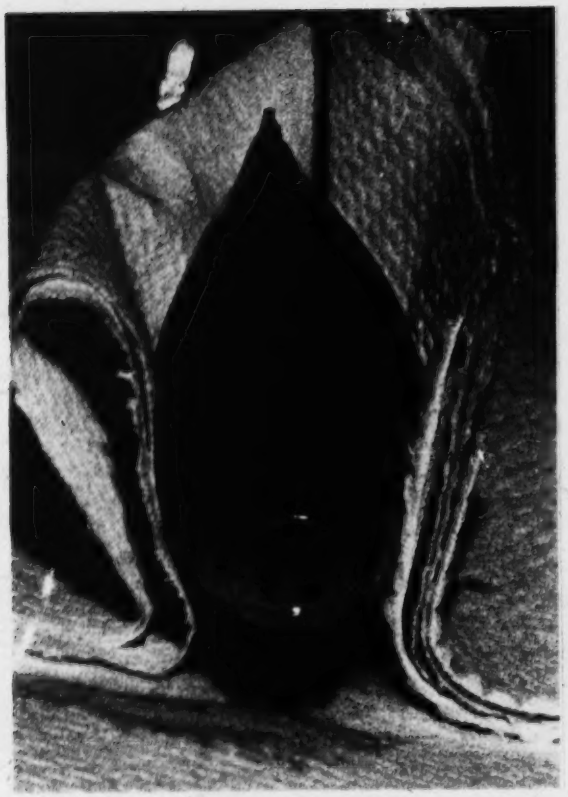
Table 13 (cont.)

Time, min	Dose; Route	Animal Weight, g	Event or Observation
0	20 mg/kg; IP - extract	24	Dosed.
3			Dead. Mouth contracted open, body rigid.
0	5 mg/kg; IP - extract	28	Dosed.
2			Pectoral fins outstretched. Mouth contracted open.
5			Dead.
0	2.5 mg/kg; IP - extract	22	Dosed.
4			Equilibrium problems.
10			Dead. No mouth contracture.
0	1.25 mg/kg; IP - extract	23	Dosed.
40			Normal, only slight effects.
0	640 mg/kg; oral - cells	25	Dosed.
3			Equilibrium problems.
8			Dead. Mouth contracted open.
0	320 mg/kg; oral - cells	25	Dosed.
15			Dead. Mouth contracted open.

Table 13 (cont.)

Time, min	Dose; Route	Animal Weight, g.	Event or Observation
0	160 mg/kg; oral	24	Dosed.
20			Dead. No mouth contracture.
0	80 mg/kg; oral	20	Dosed.
40			Normal, only minor effects.

PLATE 6. Typical response of a goldfish to an oral MLD of lyophilized cell suspension of *Anabaena flos-aquae* NRC-44-1. Note mouth contracted open.



possibility that the toxin which has been released into the surrounding water could be absorbed across the gills and cause paralysis. Goldfish placed in a live culture of toxic *An. flos-aquae*, a lyophilized aqueous cell suspension (1 mg/ml) or in an aqueous extract (6 µg/ml) failed to show any effect for up to eight hours. This indicates that fish would have to ingest toxic *An. flos-aquae* for poisoning to occur. Goldfish do not usually feed on planktonic blooms. They normally feed on insects or bottom detritus, hence should not normally be susceptible to *Anabaena* poisoning.

6. Summary. The laboratory experiments which have just been described allow some general conclusions to be drawn about symptoms and MLD's for poisonings by toxic waterblooms of *An. flos-aquae*. To relate these findings to the field situation requires some values for biomass concentrations and probable doses of natural blue-green blooms, especially *Anabaena*. A number of field observations and sample collections from natural blooms of *Anabaena flos-aquae*, *Anabaena circinalis*, *Microcystis aeruginosa* and *Aphanizomenon flos-aquae* were made which have led to the following general conclusions about blue-green bloom appearance and concentrations:

1. Growth within the water column varies from a trace to 5 mg/ml (dry weight).
2. Surface accumulation due to flotation of filaments is 5 to 10 mg/ml.
3. Heavy surface accumulation followed by wind concentration near shore and on shore in small pockets of water is 10 to 40 mg/ml (Plate 1B).

4. In some cases where heavy wave action deposits the bloom on shore and the algae become "paste-like", concentrations on the order of 70 mg/ml are attained (Plate 1C).

The condition where most consumption of bloom is likely to occur would be the heavy surface accumulation with concentrations between 10 and 40 mg/ml. Thirsty livestock would not be expected to consume concentrations of algae on the shore. With ducks which ingest algae while watering or when feeding on other aquatic plants, the algae concentration may not be as great and would be closer to 10 mg/ml. For deaths to occur the bloom would have to consist, predominantly, of *An. flos-aquae* having close to optimum toxicity (MLD IP mouse = 60). In the case where very heavy concentrations occur and where decomposition of cells could release some or all of the toxin from the cells, the MLD could approach 40 mg/kg but would not be expected to exceed this based on current knowledge of strain toxicities. Based on the information now available about susceptibility of animals, and the composition, concentration and toxicity of waterblooms of *An. flos-aquae*, the estimated dosages, taken orally, of blooms having an MLD IP mouse = 60 to cause death of mice, rats, ducks, goldfish and calves have been calculated and are summarized along with the symptoms to be expected in Table 14.

The volumes of waterbloom necessary to cause death as given in Table 14 are within the amounts normally ingested by these animals in the field. In the case of a low-concentration bloom calves might not be able to consume as much as would be required, but they could

TABLE 14

Summary of oral and IP MLD's, symptoms and estimated volumes of field dosages necessary for acute poisonings of five species of animals by waterblooms of *Anabaena flos-aquae* of low and high concentration, assuming a maximum probable toxicity of MLD IP mouse = 60.

Animal, Weight	MLD, mg/kg		Symptoms of Death	Estimated volumes of waterbloom necessary for death by the oral route (ml)	
	Oral	IP		Low Concentration (5 mg dry wt./ml)	High Concentration (40 mg dry wt./ml)
Mice 25 g	1,800	60	Twitches, convulsions, gasping respiration; survival four to five minutes	9.0	1.1
Rats 300 g	1,500	60	Twitches, convulsions, gasping respiration; survival 10 to 15 minutes	90.0	11.2
Ducks Mallard 1,000 g	350	50	Legs extended, neck contracted back over wings, gasping respiration; survival 15 to 20 min.	70.0	8.8
Goldfish 30 g	120	120	Body muscle contracture, loss of equilibrium, and in some cases mouth contracted in open position; survival 10 to 15 minutes	0.7	0.09
Calves 60 kg	360 to 480	---	Staggers, twitches, convulsions, gasping respiration; survival 10 to 15 minutes, estimation	4,320 to 5,760	540 to 720

certainly consume enough of a high-concentration bloom. With reference to the earlier reports of algae poisoning given in Table 8 (especially the symptoms recorded for birds) and to Table 11 which describes symptoms of death in ducks dosed in the laboratory it is evident that the symptoms are similar. It is implied that these early reports of algae poisonings by *An. flos-aquae* or by blooms containing *An. flos-aquae* involved a toxin that was the same or similar to the VFDF in NRC-44-1. However, results of experiments with mice given doses of toxic strains (A-113, A-123, A-128) of *An. flos-aquae* from Beaverhill Lake indicate that other toxins may be present in *Anabaena*. MLD's for these strains ranged from 60 to 120 IP mouse, which is close to that for NRC-44-1 but the survival times are longer and the symptoms are different. Death occurs in one to one and one-half hours with no twitchings, convulsions or gasping respiration. The animal shows only lethargy beginning at about one-half hour after injection until death. Autopsy shows no significant tissue degeneration except perhaps for some slight mottling of the liver. This suggests a microcystin-like effect (Konst, *et al.*, 1965) except that *Microcystis* causes twitches and convulsions just prior to death (three-quarters to one and one-half hours after injection) with pronounced mottling of the liver characteristically seen upon autopsy. An axenic clone of this *Anabaena* (A-113-9) shows a constant level of toxicity (MLD IP mouse = 80). It has not yet been established how much bacteria effect the toxicity of the strain or whether there were toxic and non-toxic clones within the parent colony isolate (A-113).

E. Pharmacology of the Toxin from *Anabaena flos-aquae* NRC-44-1

1. General. The pharmacology of a toxin refers to its mechanism of action as determined by using selective blocking agents and selected animal tissue preparations and comparing the action of the toxin with that of drugs having a known mechanism of action. In preliminary experiments in which the blood pressure and heart rate of rats was measured, lethal IP injections of the cell suspension or toxin extract resulted in a rapid drop in blood pressure which was not paralleled by a decrease in heart rate until death had almost occurred. Observations from the toxicology experiments indicated that death by the toxin was due to respiratory arrest. This respiratory arrest could be caused by peripheral neuromuscular blockade, by effects on the central nervous system (CNS) or a mixture of both. The CNS effects could be either directly on the respiratory center or by interfering with impulses to this area. To determine the mechanism of action of the toxin, a series of pharmacological experiments were undertaken. Outlines of the basic procedures for measuring the physiological parameters and the procedures for obtaining the muscle preparations are given in Appendixes as indicated for each test.

2. Effects of the Toxin on Respiration, Heart Rate, Arterial Blood Pressure and Muscle Contractions of the Rat. In preliminary pharmacological experiments rats were used as the test animal because of their availability and similarity to mice which were used as

the standard bioassay organism. Tests in which respiration, blood pressure and heart rate were measured confirmed that respiratory arrest was the cause of death. The pattern of death in these tests was:

1. Respiratory arrest occurred first.
2. Blood pressure began to fall just before respiratory arrest.
3. Heart rate began to falter.
4. Cardiac arrest occurred.

In these experiments artificial respiration (20 to 30 cycles per minute) restored normal heart rate and blood pressure. It was on the basis of these tests that the calves were dosed and maintained on artificial respiration.

In order to determine whether the toxin had any peripheral neuromuscular blocking effects experiments in which heart rate, blood pressure (femoral artery), respiration (tracheal cannula) and muscle contractions (anterior tibialis via sciatic nerve stimulation) were monitored. The condensed results of one of these experiments are given in Figure 4. The basic pattern of death was reduced blood pressure and respiratory rate followed by loss of contractions in the muscle. Heart rate was reduced toward the end of the death cycle. Loss of muscle contraction corresponded fairly closely to the decrease in respiration. It did not completely parallel the decrease in respiration because stimulation of the anterior tibialis was artificial and because of differences in susceptibility to neuromuscular blockade shown by various muscle groups (Cheymol and Bourillet, 1972c). The increase in heart rate after injection is an artifact with the same result seen if saline was injected. As can be seen, artificial respiration

FIGURE 4

Survival time and physiological pattern of an anesthetized rat injected IP with an MLD of the toxin extract. Top is respiratory rate and pattern, with inspiration below the midline and expiration above. Second from top is contraction of the anterior tibialis muscle due to square-wave pulses of one per two seconds to the sciatic nerve. Second from bottom is blood pressure via femoral artery. Bottom is average heart rate from the pulse pressure of the blood pressure transducer output.

A. Normal pattern before injection of the toxin. Respiration 75/min.

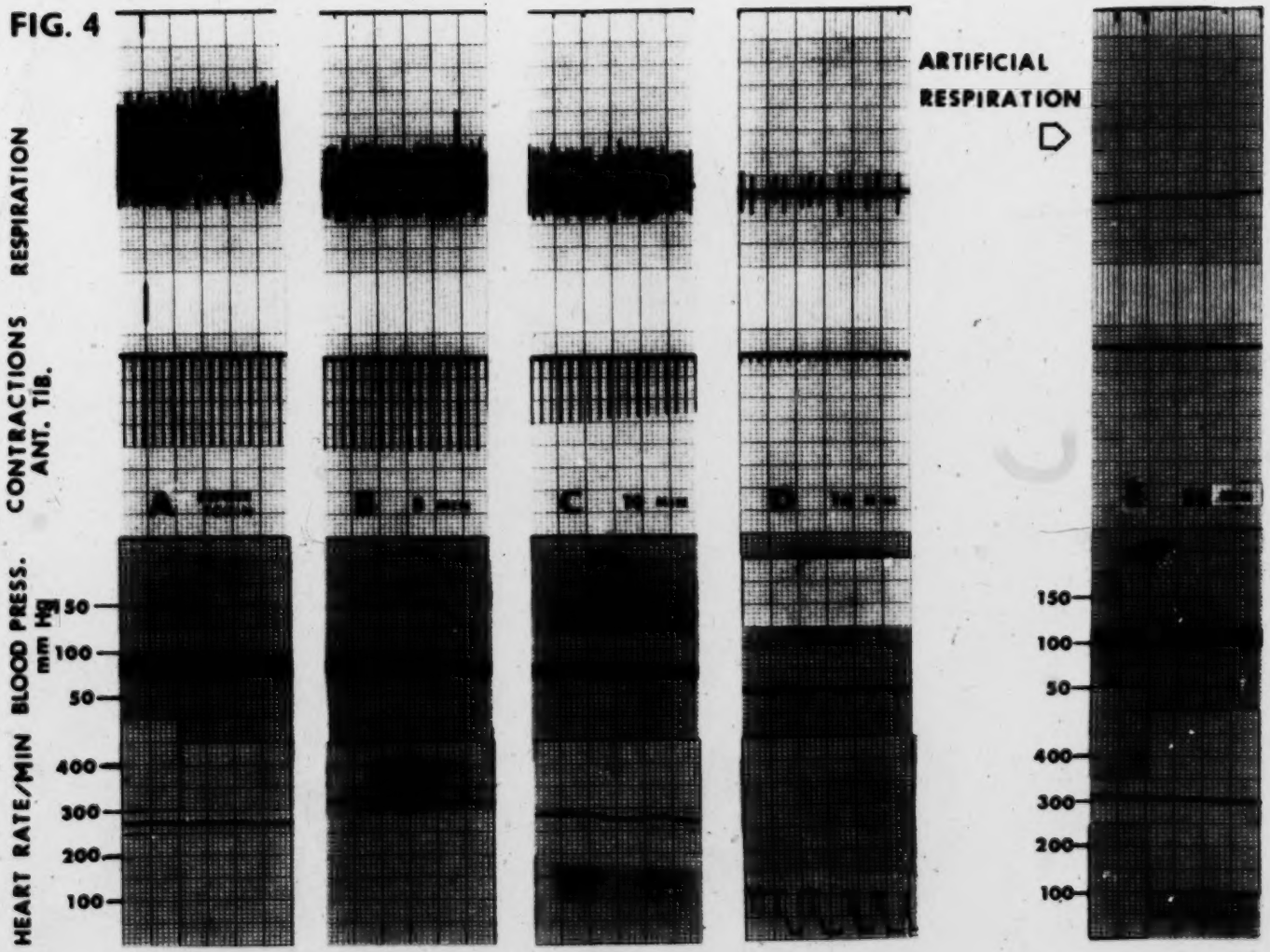
B. Five minutes after injection of the toxin. Respiration 40/min. Slight increase in muscle contraction is due to movement of baseline.

C. Ten minutes. Respiration 35/min. Muscle contraction decreasing indicating neuromuscular block.

D. Fourteen minutes. Respiratory block almost complete. Irregular heart rate is due to inadequate pulse pressure output from blood pressure transducer.

E. Twenty minutes. Restoration by artificial respiration of blood pressure and heart rate after respiratory collapse in sixteen minutes. Artificial respiration (not recorded) maintained for eight hours with no return of spontaneous respiration or muscle contraction.

FIG. 4


RESPIRATION
CONTRACTIONS ANT. TIB.
BLOOD PRESS. mm Hg
HEART RATE/MIN
150
100
50
400
300
200
100
ARTIFICIAL RESPIRATION
A
B
C
D
E

restored normal heart rate and blood pressure. Heart rate was slightly higher than control due to slight hyperventilation of the animal. Neither muscle contraction nor spontaneous respiration returned for up to eight hours of artificial respiration. The basic setup for the experiment is pictured in Plate 7. In experiments of this type no effect was seen from IV (femoral) injection of anticholinesterases (neostigmine (1 µg) or edrophonium (5 µg)). There was also no reversal of the toxin when the muscle was given tetanic stimulations (200 pulses per second). These two negative results are evidence for a depolarizing mechanism of action. Anticholinesterases are effective against competitive neuromuscular block as occurs from d-tubocurarine (Koelle, 1970b; Bowman and Webb, 1972).

The results of the *in vivo* rat experiments indicated that the toxin had a peripheral neuromuscular blocking effect that resulted in respiratory arrest. Since, at the concentration used, artificial respiration restored normal heart rate and blood pressure, a CNS effect for the toxin was not suggested but cannot be ruled out. It is known that other tertiary amines such as the erythrina alkaloids can cross the blood-brain barrier at normal clinical concentrations (Koelle, 1970b). At high concentrations or with intraventricular injections, curare does have CNS effects and this may also be the case with *Anabaena* toxin. These experiments also did not exclude the possibility that the toxin acted through a direct muscle block, even though there are very few natural toxins that have this type of action, nor did it exclude the possibility that the toxin has a tetrodotoxin-like effect (Figure

PLATE 7. General experimental setup for monitoring respiration, blood pressure and muscle contractions in the rat.

1). However, vagal stimulation (15 volts, 2 pulses/sec) leading to cardiac arrest was not blocked by injection of the toxin. If the toxin had an effect on nerve conduction or Ach release, vagal stimulation would not have caused cardiac arrest. This suggested that the toxin was either specific to the nerve-muscle junction or that it had no tetrodotoxin-like effect.

An aspect of the death pattern which was attributed to physiological changes due to hypoventilation was the altered electrocardiogram (ECG) cycle when respiratory arrest was almost complete (Figure 5). The loss of the P-wave from the pattern indicated loss of atrial depolarization. This was at first attributed to the toxin however artificial respiration restored the normal cycle. It was concluded that the physiological changes caused by insufficient oxygen (decreased blood pressure, altered blood pH, higher PCO_2, *etc.*) were responsible for this arrhythmic pattern (Owen, 1973).

The toxin could now have either a post-synaptic or pre-synaptic effect (see Figure 1). To check further the effects of the toxin on skeletal muscle, rat phrenic nerve diaphragms were prepared (Appendix VI). This muscle preparations is commonly used to evaluate activity of neuromuscular blocking agents. The tests conducted using this tissue preparation were done to:

1. Indicate a range of activity for the toxin.
2. Compare its activity with other neuromuscular blocking agents.

The results of some of these tests are given in Figure 6. These indicate several things:

FIGURE 5

Electrocardiogram (equivalent to human Lead II) of an anesthesized rat, treated with a lethal IP dose of toxin extract.

A. Before injection. Normal PQRST complex. Heart rate approximately 250/min.

B. Fourteen minutes after injection of toxin showing altered pattern when respiratory collapse is almost complete. P-wave is lost. Heart rate about 180/min.

C. Twenty minutes after injection of toxin showing normal pattern restored with artificial respiration. Heart rate about 350/min.

A, B and C correspond to A, D and E of Figure 4.

FIG. 5

BEFORE TOXIN

RESPIRATORY COLLAPSE

ALMOST COMPLETE--

P-WAVE LOST

ARTIFICIAL RESPIRATION

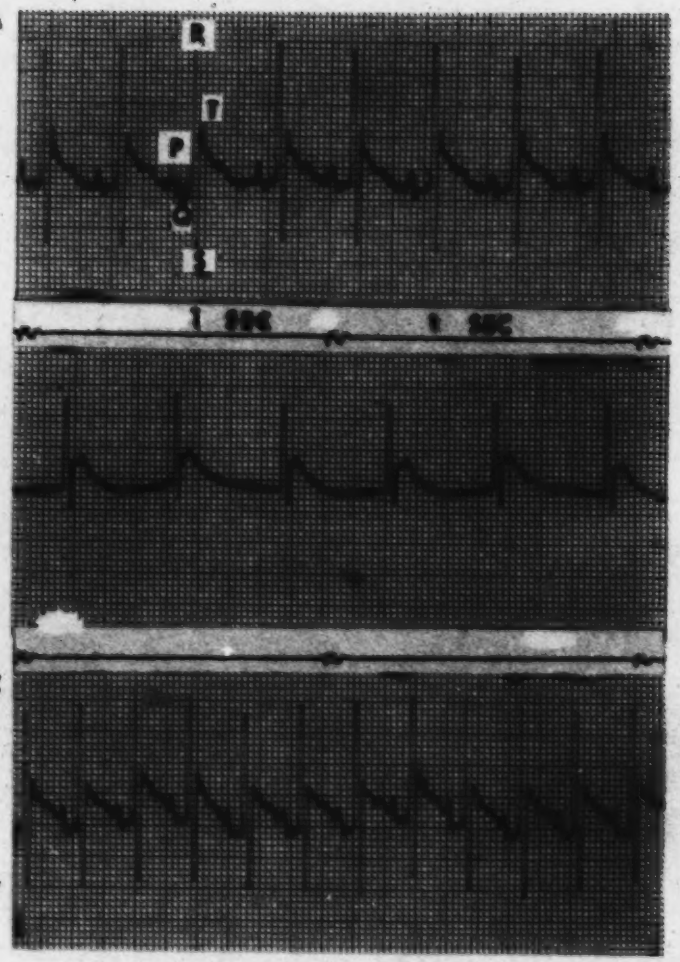


FIGURE 6

Effects of toxin extract (TE) (MLD IP mouse = 2.5), d-tubocurarine (DTC), tetrodotoxin (TTX) and antagonism by edrophonium (EC) on contractions of the rat hemidiaphragm.

A. TE at arrow (5 µg/ml). Muscle block complete in two minutes.

B. TE at 1 (15 µg/ml). EC at 2 (5 µg/ml). Some reversal of muscle block but it is not permanent.

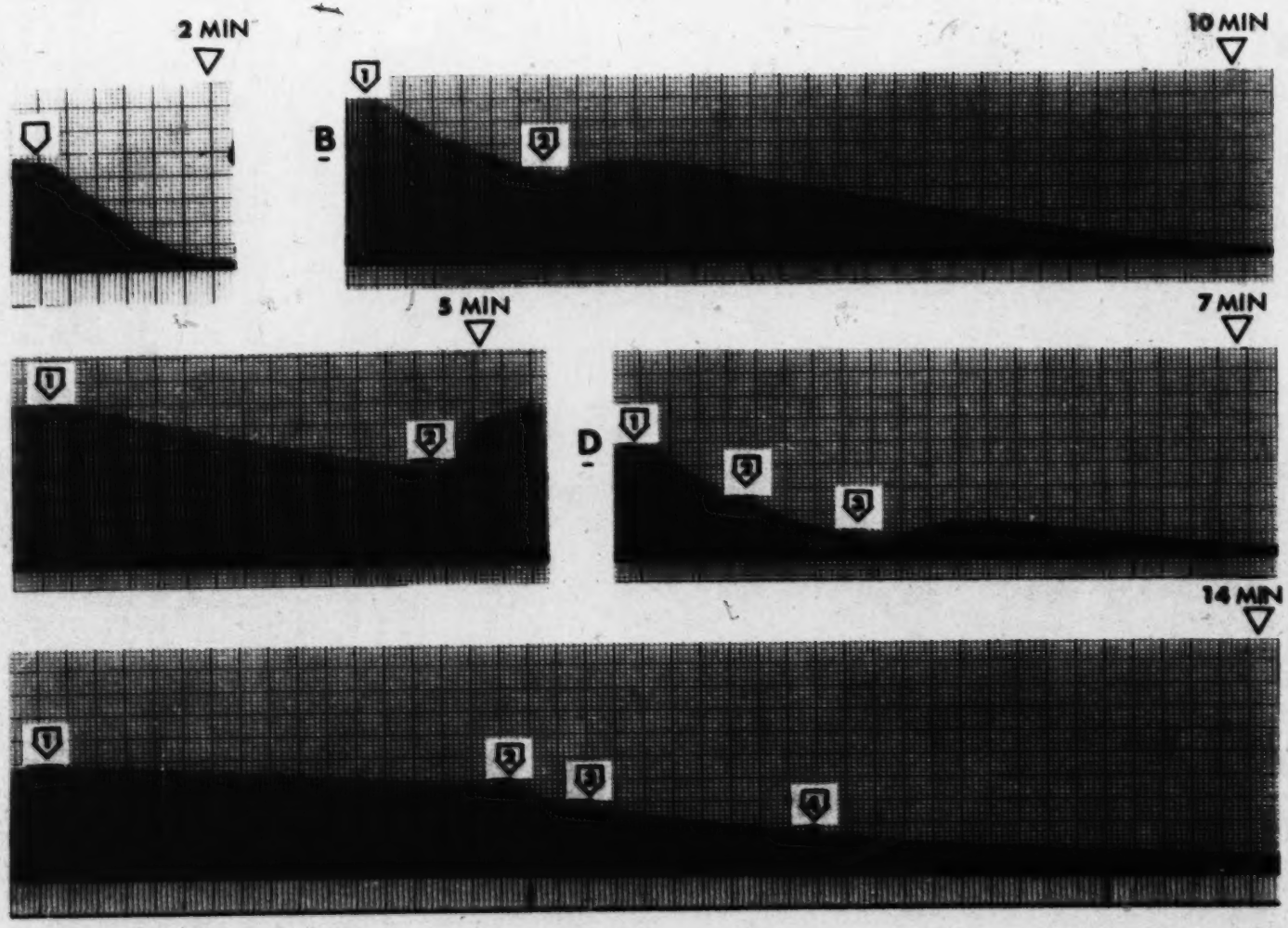
C. DTC at 1 (4 µg/ml). Normal muscle contraction restored at 2 by addition of EC (5 µg/ml).

D. Prior treatment at 1 with DTC (2 µg/ml) does not protect against TE added at 2 (1.5 µg/ml). EC at 3 (5 µg/ml) shows similar pattern to B.

E. TTX at 1 (0.05 µg/ml) and at 2 (0.025 µg/ml) is not affected by EC at 3 and 4 (total of 10 µg/ml).

A and E are with the same hemidiaphragm washed for one hour between tests with Krebs saline. B, C and D are with another hemidiaphragm. D followed one hour washing of B in which normal contraction had not returned before D was done.

FIG 6



1. It confirms again the neuromuscular blocking activity of the toxin at dose levels within the range of other neuromuscular blocking compounds.
2. The effects are reversible with time by washing the muscle preparation with saline.
3. The toxin extract does not resemble curare in that it is not completely reversed by the anticholinesterase edrophonium. This implies a depolarizing or mixed type of activity.

Interruption of transmission of the nerve impulse at the skeletal neuromuscular junction can be classified as either competitive (curare) or depolarizing (decamethonium) (Koelle, 1970). This can best be explained by a short review. Acetylcholine concentrations in the synaptic gap are regulated by the naturally occurring enzyme cholinesterase. Anticholinesterases such as edrophonium or neostigmine act to either prolong the life of the transmitter or to inhibit cholinesterase activity. Thus curare, which blocks the post-synaptic receptor site of acetylcholine, has its activity reversed by anticholinesterases (increase of the local acetylcholine) (Figure 6C) (Bowman and Webb, 1972). The temporary reversal of the toxin induced muscle block by edrophonium can be likened to the depolarizing effect exhibited by high concentrations of acetylcholine on cat soleus muscle in the presence of anticholinesterases (Bowman and Webb, 1972).

4. Under the conditions of these diaphragm preparations curare did not provide protection to the muscle when given before the toxin (Figure 6D). Curare antagonism is given as a characteristic for depolarizing drugs (Koelle, 1970).

5. Figure 6E indicates that the activity of the toxin is not like that of tetrodotoxin on rat hemidiaphragm.

Initial potentiation of muscle contractions (fasciculations) are a characteristic of depolarizing drugs in single innervated muscle such as used in the rat. There was, however, no clear indication of this in the hemidiaphragms or anterior tibialis muscle preparations tested. This can possibly be explained if the toxin has a depolarizing effect. The partial reversal of the muscle block by edrophonium could be due to the muscle being in Phase II of a depolarizing blockade having passed through or not showing a *in vitro* Phase I effect (Cheymol and Bourillet, 1972c). It was concluded that rats are not a particularly good preparation for this response, and to be properly tested the literature in this area indicates that cat soleus or anterior tibialis muscle should be used.

3. Depolarization by the Toxin Extract on Frog Rectus Abdominus Muscle. As previously mentioned, many amphibian muscles contain multiply innervated fibers. Since the depolarization produced by acetylcholine or similar drugs is not associated with a refractory period, muscle shortening persists throughout the period of contact and its amplitude is related to the concentration (Bowman and Webb, 1972). For this reason comparison of dose response lines of the muscle preparation to acetylcholine and the toxin can verify its depolarizing activity. The results of this test are given in Figure 7. The muscle preparation is described in Appendix VII and the general

FIGURE 7

Regressions of frog rectus response on dosage for toxin extract (TE) (MLD IP mouse = 0.3), acetylcholine (ACH) separately and in combination with d-tubocurarine (DTC).

A. Dose response for TE.

B. Dose response for TE when DTC is present in the saline bath at 1 µg/ml. DTC caused a dose shift about four times that of A for the same muscle response.

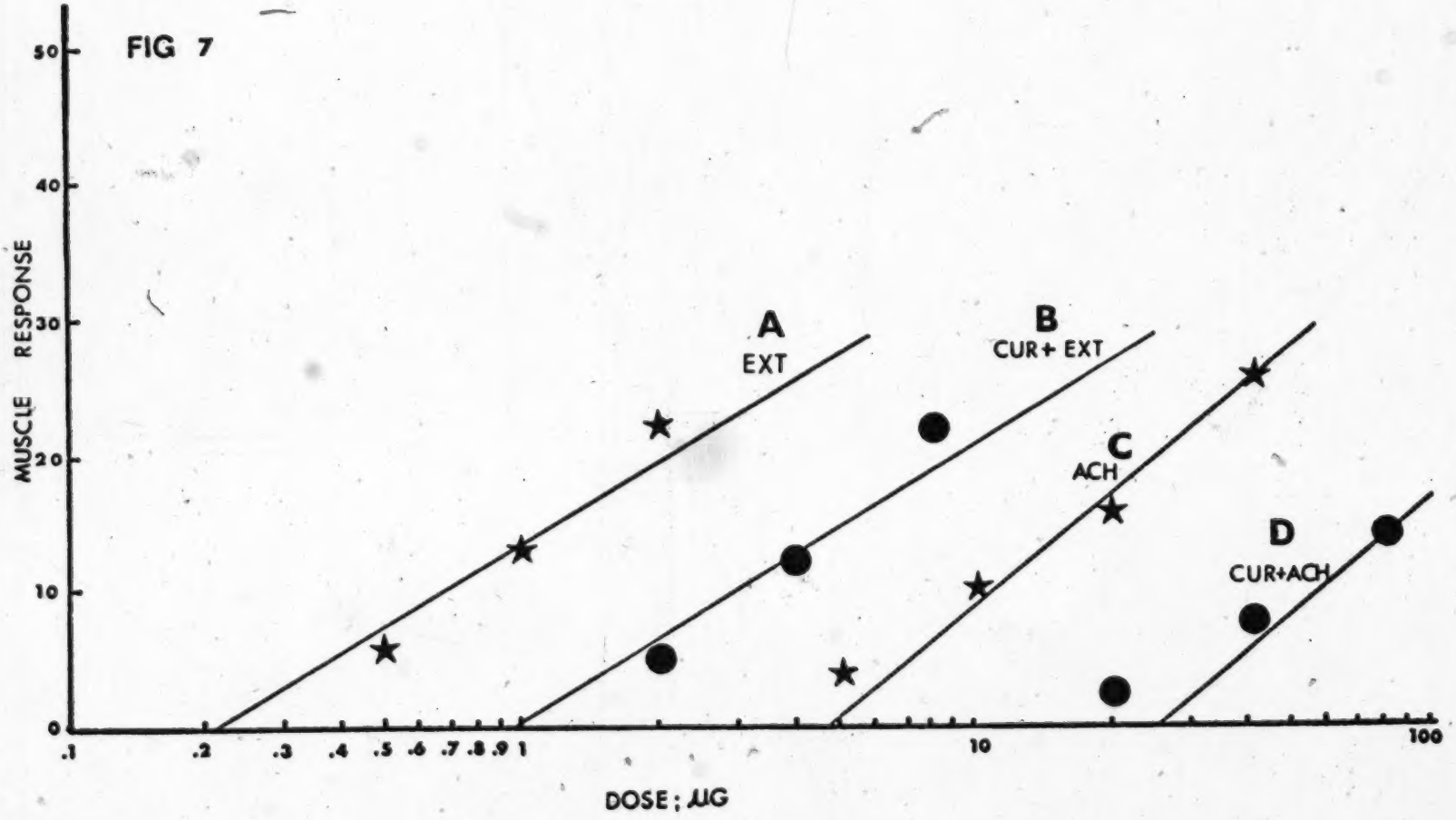
C. Dose response for ACH.

D. Dose response for ACH when DTC is present in the saline bath at 1 µg/ml. DTC also caused a dose shift about four times that of C for the same muscle response.

Since the muscle is in a 20 ml organ bath, µg/ml can be obtained by dividing by 20.

Goodness of fit test in individual lines is significant at the 90% level (Snedecor and Cochrane, 1967). Each point is an average of four values for muscle response.

FIG 7

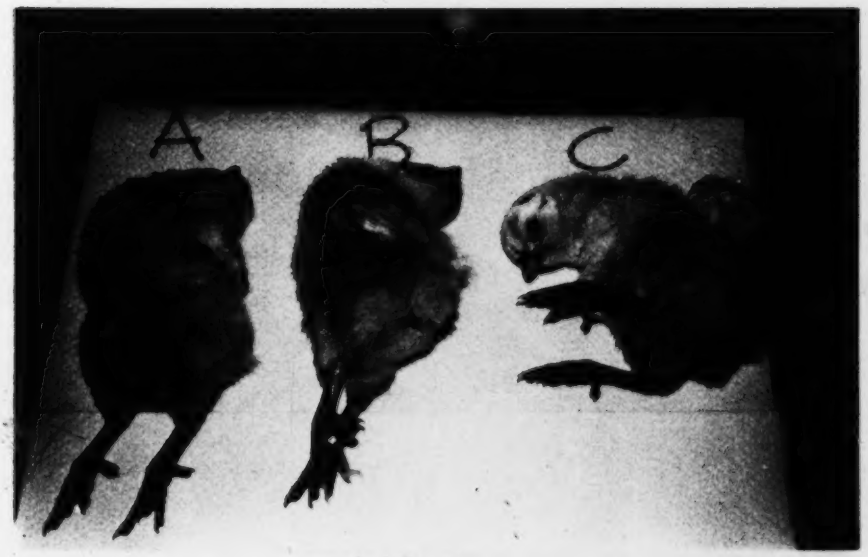

MUSCLE RESPONSE
50
40
30
20
10
0
A
EXT
B
CUR+ EXT
C
ACH
D
CUR+ACH
.1
.2
.3
.4
.5
.6
.7
.8
.9
1
10
100
DOSE ; µG

experimental setup is shown in Plate 8. The results given in Figure 7 show a parallel shift in dose response to acetylcholine and toxin extract in the presence of d-tubocurarine. The parallel shift is also qualitatively similar which implies that the toxin is specific in its active site at the nerve-muscle junction. This indicates competitive inhibition by d-tubocurarine and points to the depolarizing action of the toxin. The results also rule out a direct effect on the muscle. It also indicates that the toxin extract is about 20 times as active as acetylcholine on frog rectus in causing a depolarizing response.

4. Depolarizing Neuromuscular Blockade Produced in Chicks by the Toxin Extract. The symptoms of death for cell suspensions of the toxin given orally or IP to ducks strongly indicated a depolarizing mechanism of action. The opisthotonus observed in these ducks compares very well with the response of day-old chicks injected (IV) with the known depolarizing drug decamethonium and the toxin extract. A comparison of these two drugs with the flaccid paralysis of d-tubocurarine illustrates the point even further (Plate 9). Decamethonium (C-10) has been studied extensively as a member of the series of substances with a depolarizing mode of action. Its mechanism of action is more complex than that of d-tubocurarine since there are differences in mechanism of action among species and in the same species according to the muscle. On mammals, including the cat, C-10 produces fasciculations and a facilitation of the muscle contractions. This is the depolarizing cholinomimetic action. It is followed by such characteristics as antagonism by neostigmine (or edrophonium). This stage

PLATE 8. General setup for monitoring contractions of frog rectus in an organ bath.

PLATE 9. Typical depolarizing syndrome in day-old chicks to an IV dose of: A. decamethonium (0.2 mg), B. toxin extract (0.2 mg), C. typical flaccid paralysis due to lethal IV injection of d-tubocurarine.



of the paralysis is curarimimetic (curare-like). This is referred to as the "dual block" described by Zaimis (1953). The importance of the initial depolarizing component in comparison with the competitive component varies with species and accounts for the variation in reactions to C-10 (Cheymol and Bourillet, 1972c). In reference to the rat muscle preparations the depolarizing component was not seen but the competitive component was. On avian muscle, opisthotonus is seen with no phase of paralysis unless the dose is lethal. These contractures by C-10 are displaced from the baseline without much reduction in amplitude.

The basic action of C-10 on motor nerve terminals during what is referred to as the Phase I (cholinomimetic) block is to depolarize the post-synaptic membrane, as does acetylcholine, by a local increase in the permeability of the membrane to Na and K ions. However, cholinesterases which limit in time the effects of acetylcholine are without effect on C-10. The depolarization thus becomes prolonged and its persistence beyond a certain level (lethal doses) leads to inactivation of the transport mechanism for Na. The muscle membrane surrounding the endplate becomes electrically inexcitable. Transmission is blocked. d-Tubocurarine, which becomes fixed to the same receptors without depolarization, can prevent or antagonize this blockade (Cheymol and Bourillet, 1972c).

The tachyphylaxis observed to successive doses of decamethonium has been explained by Burns and Paton (1951) and supported by more recent studies. They suggested that C-10 entering into the interior

of the muscle fiber is an essential part of the depolarization process. Depolarization then depends on the concentration difference of the drug across the membrane. Repolarization of the membrane would occur when there was the same amount inside as outside, but the amount within the muscle would reduce the rate of entry (hence depolarization) produced by subsequent doses of decamethonium (from Bowman and Webb, 1972). Tachyphylaxis occurs during what is called the Phase II block in response to decamethonium. Phase II occurs if the dose is not sufficient for death during Phase I. It is distinguished from Phase I by a temporary return of contractions on isolated preparations. In spite of the C-10 present in the receptor region, the endplate becomes repolarized and becomes electrically excitable. During this repolarization of the membrane the receptors become less and less sensitive to depolarizing agents by the mechanism just given. This is tachyphylaxis (Cheymol and Bourillet, 1972c).

5. Depolarizing Neuromuscular Blockade Produced in the Gastrocnemius Muscle of the Duck by Toxin Extract. Certain aspects of the responses to *Anabaena* toxin should now begin to fit into this preceding discussion of depolarizing properties of C-10. Further evidence for depolarization and correlation with depolarizing drugs was provided by *in vivo* experiments with duck gastrocnemius muscle sciatic nerve preparations (Figure 8 and Appendix VIII). The data in Figure 8 is best explained by reference to some discussion of avian muscle. The Phase I and II block just discussed explain the depolarizing response of C-10 mainly in reference to focally (singly)

innervated muscle fibers such as occur in mammals. In many amphibian and avian species, muscle fibers receive multiple innervation and therefore acetylcholine-sensitive areas are widely distributed over the membrane surface. This results in a much greater sensitivity to IV injection of depolarizing drugs or acetylcholine. The greater sensitivity being mainly due to the fact that in multiply innervated fibers the drug has greater surface area available for contact. The graded depolarization produced may be sufficiently widespread to activate most of the contractile mechanism (Bowman and Webb, 1972). The result is as seen in Figure 8, and is characterized by rising baselines but essentially constant amplitude of muscle contractions. It can be said then that the VFDF toxin has similar properties to depolarizing drugs on duck gastrocnemius muscle. The competitive block produced by d-tubocurarine in the same muscle (Figure 8D) prevented depolarization of the muscle by sublethal doses of the toxin extract. However a 100 µg injection of the toxin extract followed by 20 µg of d-tubocurarine did not prevent depolarization and subsequent complete neuromuscular blockade. The results of the duck gastrocnemius and chick IV injections strongly suggest a depolarizing mechanism of action for the toxin extract. It also explains the opisthotonus observed in ducks given oral or IP doses of toxic cell suspensions.

6. Effect of Toxin Extract on Guinea-Pig Ileum. As a final pharmacological test of the toxin its effect on guinea-pig ileum was tested. This was done to check for a possible autonomic ganglionic

FIGURE 8

Effects from IV (jugular) injection of toxin extract (TE), suxamethonium (SUX) and d-tubocurarine (DTC) on mallard duck gastrocnemius muscle contractions. Muscle is stimulated via the sciatic nerve.

A. Injection at arrow of 25 µg TE (MLD IP mouse = 0.3). Rising baseline which indicates depolarization was maintained for 22 minutes before returning to control level.

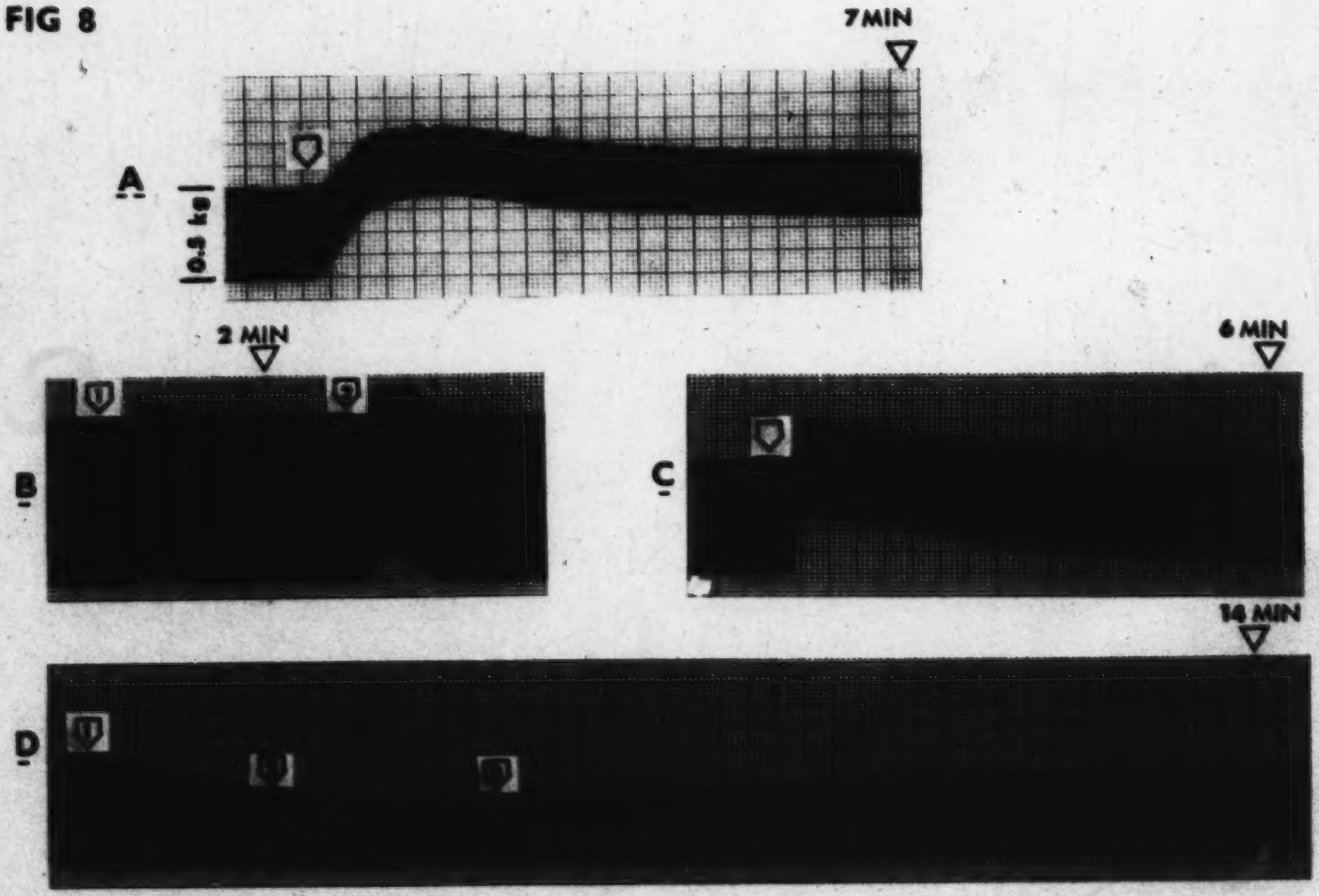
B. Injection at 1 (5 µg) and at 2 (10 µg) of SUX.

C. Injection at arrow of 25 µg TE (MLD IP mouse = 2.5). Baseline returned to control level after 14 minutes. Transducer output adjusted from B to give smaller output.

D. Injection at 1 of 10 µg DTC. Decrease in contractions indicate competitive block. Injection at 2 (20 µg) and at 3 (25 µg) of TE resulted in no depolarizing response indicating DTC protection.

0.5 Kg refers to tension exerted by contracting muscle on spring inserted in transducer. A is one muscle preparation; B, C and D are from another duck.

FIG 8



(nerve synapse) effects of the toxin. This would be in contrast to its now known effect on skeletal nerve muscle synapses. The response of guinea-pig ileum to acetylcholine (Ach) and extract would be expected to be similar to frog rectus (sustained contraction in a graded dose response manner). Because it is a smooth muscle preparation containing autonomic ganglia it would also be affected by ganglionic blocking drugs such as hexamethonium. Hexamethonium prevents post-synaptic depolarization by nicotine or DMPP (1,1-dimethyl-4-phenylpiperazinium iodide). It does not prevent depolarization by Ach because of the multiple cholinoceptive cites present in sympathetic ganglia (Voelle and Koelle, 1970). With nicotine contraction reductions, the indication is that the "muscarine-sensitive" postganglionic Ach receptors are more sensitive to acetylcholine than the "nicotine-sensitive" receptors in the parasympathetic ganglia. Muscarine-sensitive receptors or muscarinic effects (pertaining to the alkaloid muscarine) refer to the cholinomimetic effects of drugs at autonomic effector cells, including those of Ach itself. The stimulation and then blockade of autonomic ganglia and the endplate of skeletal muscle, as exhibited by high Ach, nicotine and DMPP, are termed nicotinic effects (Koelle, 1970a).

The muscle preparation is described in Appendix IX. Dose response was linear for acetylcholine from 0.025 µg to 0.8 µg (divided by 20 for concentration per ml). Linear dose response values for toxin extract (MLD IP mouse = 0.3) were observed for concentrations of 0.5 µg to 20 µg added toxin. In the presence of hexamethonium in the saline

(100 µg/ml), response of the muscle to toxin extract was blocked until a concentration of 32 µg/ml (640 µg toxin) was added. Response of the muscle to acetylcholine in the presence of hexamethonium was not affected. Hexamethonium was at a concentration which would block the stimulating effects of nicotine or DMPP (1,1-dimethyl-4-phenylpiperazinium iodide). The results implied that the toxin has some postganglionic stimulating effects in the guinea-pig ileum. This would then indicate that it is not specific for depolarization of neuromuscular junctions. It is, however, more evidence for its post-synaptic effect.

7. Summary. The results of these experiments allow several important conclusions to be drawn about the mechanism of action of the toxin from *Anabaena flos-aquae*. In mammalian muscle the toxin produces a neuromuscular block that leads to death by respiratory collapse. Artificial respiration can restore normal heart rate, blood pressure and ECG. The effects of the toxin are long-lasting. Tachyphylaxis is observed to successive sublethal doses of the toxin and can be explained if the toxin is a depolarizing drug. However not all characteristics of depolarizing drugs in mammals have been observed with the toxin, and this may be due to variability with species and muscles within species. It is also possible that the toxin does not possess all the classical characteristics of the depolarizing drugs. In avian (ducks) and amphibian (frog rectus) muscles and in general observations on chicks, the toxin presents

a very characteristic depolarizing response. On frog rectus a depolarizing response to the muscle was induced by both acetylcholine and toxin extract. Both compounds were competitively inhibited in a parallel manner by d-tubocurarine. The toxin extract has depolarizing properties at concentrations less than that of acetylcholine on frog rectus. In duck gastrocnemius the toxin-extract-induced depolarization could be inhibited by d-tubocurarine within at least some concentration ranges. An experiment on guinea-pig ileum suggests that the toxin may also act at sites other than the neuromuscular junction. On the animal tissues used in these experiments the toxin extract has an activity at least as great as the depolarizing drugs now known.

DISCUSSION

Anabaena flos aquae is a species of freshwater blue-green alga that has come to be called one of the "bloom" formers. Other species within the genus can also form blooms but *An. flos-aquae* has the distinction of at times forming toxic blooms. The reported occurrence of toxic *An. flos-aquae* blooms goes back about 50 years while toxic freshwater blue-green blooms have a reported history of about 100 years. While *An. flos-aquae* can form blooms during all of the summer and fall months if given proper environmental conditions, its usual sequence in bloom succession is late spring and early fall (Hammer, 1964). It has been known for some time that there are toxic and non-toxic strains (colonies) within a bloom (Gorham, *et al.*, 1964). Laboratory studies on the physiology of optimum toxin production in toxic strains correlates with conditions of good growth. Toxin variability of colony isolates in the laboratory can now be explained entirely on the basis of toxic and non-toxic filaments within the colonies and the presence of bacteria. Because of this it should now be possible to produce standard tables of MLD's by altering the ratios of toxic and non-toxic filaments. These tables could be used with the MLD's of toxic waterblooms to predict relative numbers of toxic and non-toxic filaments. Appropriate weighting factors would have to be included for the presence of bacteria and amount of decomposition in the bloom. Using a laboratory example it is probable that hetero-

geneous colony isolate A-52-4{m} which had an initial MLD of 640 (Table 7) consisted of toxic and non-toxic filaments in a ratio of about 1:4 or 1:8.

Certain bacteria seem to be able to inhibit toxin production by some as yet unknown mechanism without affecting algal production. The role that these bacteria play in the regulation of toxicity in natural blooms is not too clear but it appears to be secondary, Since they do not seem to be able to render toxic laboratory cultures non-toxic and it is doubtful whether they do more than partially reduce toxicity under natural conditions. For deaths of wildlife and livestock to occur in the field the bloom must be concentrated and consist mainly of toxic *An. flos-aquae* filaments to provide enough toxin in the quantities that different animals are likely to ingest. The primary factor then in determining toxicity of water-blooms is their genetic constitution. Modifying secondary factors include toxin-inhibiting and toxin-degrading bacteria as well as a number of physical factors suggested previously. As has already been pointed out, accessibility and susceptibility by animals also play an important role in determining animal losses. It is now evident however that given optimum toxicities and susceptibilities to acute dosages, amounts consumed by normal drinking or feeding habits of the animals most effected are well within the limits for causing death. As inferred by observations on mallard ducks and calves, waterfowl and ruminant livestock appear to be more sensitive to the toxin than other animals. Monogastrics, which includes man, could be

expected to be more resistant. However, depending on age, size, condition and circumstance even these animals are susceptible, although the records indicate that it is not as likely.

The tachyphylaxis observed in one of the calves, a duck and in earlier experiments on rats is a feature of the dual blockade mechanism of depolarizing drugs described by Zaimis (1953 and 1959). It could explain why some animals appear to have more resistance to a toxic waterbloom than others having access to the same source of water. If animals are watered in an area where the toxic bloom is accumulating it is possible that, depending on an animal's watering habits, it could ingest more than a lethal dose and not die, but an animal ingesting an amount equal to a lethal dose in one bolus would collapse with respiratory arrest. While it is not known how long resistance to sublethal doses lasts this implies that animals which have continual access to an accumulating toxic bloom would be less susceptible than animals which have access only after the toxic bloom is well formed. The opisthotonus that would be observed in ducks or other waterfowl and birds can be taken as very characteristic of the toxin. This is because there is no other freshwater or marine plant toxin which is known to have depolarizing properties. The neck contracture and leg extension of ducks has some of the visual properties of fowl botulism "limber neck". Fowl botulism, however, which is caused by the bacterium *Clostridium botulinum* (type C) produces a flaccid paralysis with floppy neck and feet not extended. The nictitating membrane is also not paralysed in *Anabaena* poisoning as it is with

botulinum (Graham and Bouthton, 1923; Kalmbach, 1968; Enright, 1971). It is suspected that in some cases where this alga was responsible for deaths of ducks and other birds the neck thrown back over the shoulder could resemble limber neck. It is only to be emphasized here that the two are physiologically distinct.

The toxin from *Aphanizomenon flos-aquae* which was responsible for large fish kills (Sawyer, Gentile and Sasner, 1968) is reported to be an endotoxin (Gentile and Maloney, 1969). Sawyer, *et al.* (1968) report that white sucker, guppy and pumpkinseed fish placed in lyophilized cell suspensions containing 4 X 10^5 cells/ml of toxic *Aphanizomenon* resulted in survival times ranging from 15 to 240 minutes for these species. Gentile, *et al.* (1969) report that lyophilized culture supernatant injected IP into mice had no effect while injection of lysed and unlysed cells killed mice in 1.25 and 14.5 minutes, respectively. Fish (golden shiner) were killed in an aqueous medium containing a minimum effective toxin extract (LD_{100} IP mouse = 8 mg/kg) concentration of 25 µg/ml. Survival times were however about 60 hours. Thus they say that the toxin is an endotoxin and that fish kills were a result of toxin release from decomposing cells following copper sulphate treatment of the toxic waterbloom. The results of *An. flos-aquae* toxin on goldfish indicate that the toxin, even though it is an exotoxin, is not readily absorbed across the gill membranes. There may, however, have been more of an effect if the fish had been left for a longer time. From the experiments conducted to date, however, there is an indication that *Aphanizomenon* toxin is more of a threat to fish

than *Anabaena* VFDF toxin. There do not appear to be any reports of depolarizing drug effects on fish so that the mouth contracture and general body rigidity observed should be fairly characteristic of the toxin just as it is in ducks.

The structure of the toxin[1] (Figure 9) which was first proposed as being a tertiary amine with a characteristic absorbance at 229 nm by Stavric and Gorham (1966), has been deduced on chemical and spectroscopic grounds by R. Pike, O. E. Edwards and co-workers (personal communication, NRC Ottawa) and confirmed by Huber (1972). The structure is a novel one not only for a natural alkaloid but also for its depolarizing properties. It most closely resembles L-cocaine (Figure 9) of the tropane series (Huber, 1972). Cocaine, however, has a seven-membered ring and the toxin has eight. The extra carbon in the ring is joined by a double bond which seems to give the structure its biological activity. Further evidence which confirms the proposed structure has come from a crude synthetic mixture supplied recently by O. E. Edwards for pharmacological testing. This crude mixture was estimated to have about eight per cent active ingredient on the basis of absorbence at 229 nm. Bioassay (IP) of the mixture with male mice (18 to 23 g) resulted in typical VFDF symptoms and death

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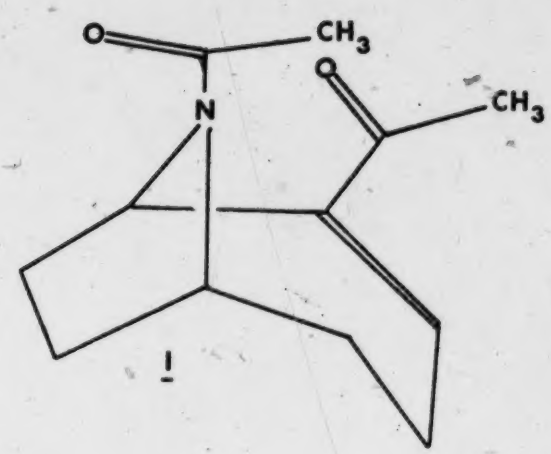
[1]For this study the term adopted for the toxin from NRC-44-1 has been *Anabaena* VFDF or *An. flos-aquae* VFDF. This was done to distinguish it from what appears to be another toxin or toxins within this species. It will continue to be a useful term until more detailed structure-function relationships are made between the toxin and other depolarizing drugs or neuromuscular toxins.

FIGURE 9

Chemical structure of *Anabaena flos-aquae* NRC-44-1 VFDF toxin (I), (Huber, 1972); L-cocaine (II); decamethonium (III); succinylcholine (suxamethonium) (IV); d-tubocurarine (V); and nicotine (VI).

FIG 9



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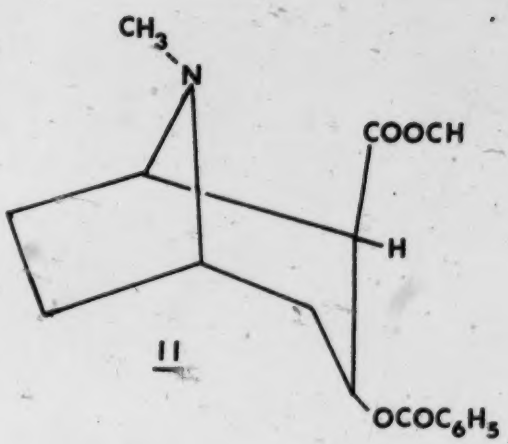

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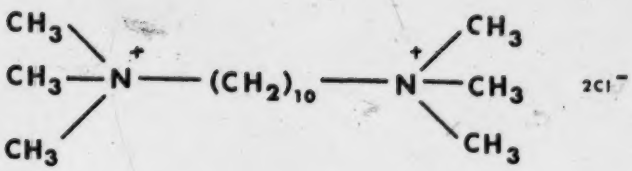

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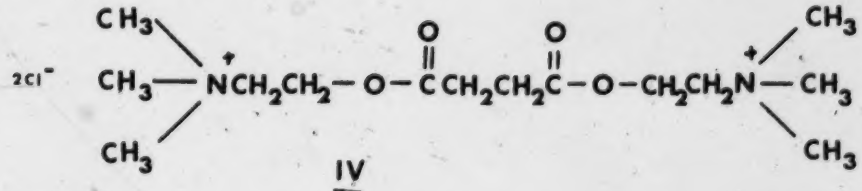

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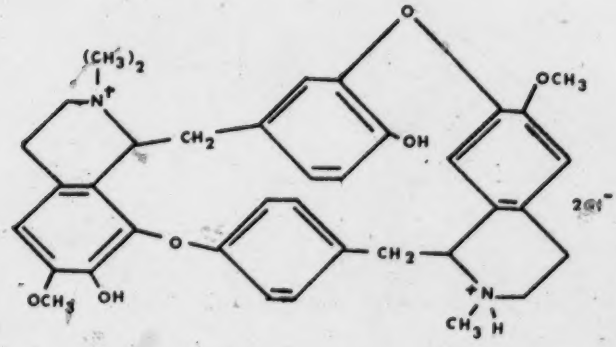

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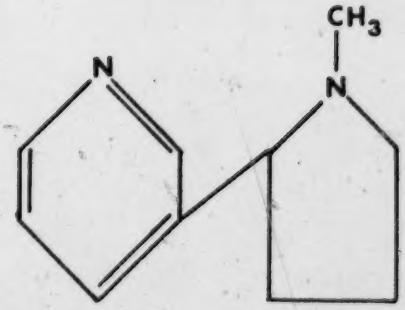

VI

times (MLD = 5.0 mg/kg). IV injection of the mixture as well as decamethonium into chicks produced similar responses (opisthotonus) (Plate 10). Based on the estimated eight per cent active ingredient the MLD IP mouse was 0.4. This compares favorably with the toxin extract which has an MLD of about 0.3 for the most active fraction. This is, therefore, evidence that the toxin extract used in these studies was fairly pure. Figure 9 also gives the structure of some other neuromuscular toxins. It is evident that VFDF does not resemble the other known depolarizing drugs in structure.

Another interesting feature of the toxin is that it is absorbed orally. Neither decamethonium nor suxamethonium have much activity even at very high doses when given orally. They are most effective when given IV. d-Tubocurarine is also not readily absorbed orally but is most effective when given intramuscularly or intravenously (Koelle, 1970b). It should also be noted that there appears to be only a few other natural products which have depolarizing properties. One is nicotine whose structure is very different from *Anabaena* VFDF. In terms of marine or freshwater toxic algae (Shantz, 1971; Gentile, 1971; and Shilo, 1971b) *An. flos-aquae* VFDF toxin is the only one to possess depolarizing properties.

The stabilization of toxin production in laboratory cultures of toxic *An. flos-aquae* and an effective extraction procedure have allowed good progress to be made in understanding the toxicology and mechanism of action of the toxin. At this point the work has shown a very probable mechanism of action but more needs to be learned

PLATE 10. Typical paralysis in chicks from: A. decamethonium, B. d-tubocurarine and C. synthetic toxin. Note similarity to Plate 9. All injections were 0.2 mg.

about its site or sites of action. There is no adequate chemical antagonist of the toxin at this time. This is because it is a depolarizing drug for which no adequate antagonists are known. Competitive neuromuscular blocking drugs such as d-tubocurarine, on the other hand, are effectively antagonized by anticholinesterases. Some of the earlier reports on algal poisonings in which *An. flos-aquae* was present report some success in antagonizing the toxic factor. This was done for cows by IV injections of thionitrite tablets (sodium nitrite 10 g; sodium thiosulfate 50 g) (MacKinnon, 1950; O'Donoghue and Wilton, 1951). Rosé (1953) reported some success with oral flushings of a solution of potassium permanganate and Epsom salts. In the MacKinnon report the alga or algae responsible were not identified. The O'Donoghue and Wilton report suggests a *Microcystis* sp. The Rose report identifies *An. flos-aquae* as the toxic organism. In the case of *Microcystis* it is not known what effect these procedures would have on the polypeptide toxin. If the first report was dealing with an *Anabaena* poisoning and considering what is now known about the toxin, any benefit from the thionitrite treatment may have been the result of reducing and detoxifying any residual toxin left in the digestive and circulatory systems. If an animal received a lethal bolus of toxic *Anabaena* death would come too quickly and by a mechanism for which these compounds would have little effect.

While it may now seem that much is known about toxic *An. flos-aquae*, as with most science, the work is never complete. Several

important areas of investigation remain. First there is the problem of understanding the mechanism or mechanisms which result in toxic strains becoming dominant over non-toxic strains in relation to the formation of toxic blooms. There is then the area of understanding more fully the mechanism and site of action for the toxin. This is not only in regard to a possible antidote but also to what this particular structure and its properties can contribute to understanding certain physiological processes. The question of biosynthesis of the toxin and what it could contribute to understanding the biosynthesis of natural alkaloids in general could be studied. This may prove easier in an organism that can be cultured under more specific and controlled conditions than higher plants which are traditionally used for this type of work (Spenser, 1970). Finally there is the fascinating but perhaps arm-chair research that can be done pursuing the significance of a phylogenetically ancient organism producing a compound which possesses such intriguing properties affecting organisms of a much more recent phylogenetic origin.

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APPENDIX I

Components of ASM-1-TR Culture Solution

Component	Concentration, mM, in culture solution
$NaNO_3$	2.0
K_2HPO_4	0.1
$Na_2HPO_4 \cdot 7H_2O$	0.1
$MgCl_2 \cdot 6H_2O$	0.2
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2 \cdot 2H_2O$	0.2
$FeCl_3$	0.004
Micronutrients:	
H_3BO_3	0.040
$MnCl_2$	0.007
$ZnCl_2$	0.0032
$CoCl_2$	0.00008
$CuCl_2$	0.0000008
Tricine	0.15
Na_2EDTA	0.02

APPENDIX II

Weight of Salts in ASM-1-TR

Component	mg/liter
$NaNO_3$	170.00
K_2HPO_4	17.40
Na_2HPO_4	14.20
$MgCl_2$	19.02
$MgSO_4$	24.08
$CaCl_2$	22.20
$FeCl_3$	0.65
H_3BO_3	2.47
$MnCl_2$	0.87
$ZnCl_2$	0.44
$CoCl_2$	0.01
$CuCl_2$	0.0001
Tricine	26.90
Na_2EDTA	6.64
TOTAL	304.88

APPENDIX III

Bacteriological Media Used to Culture Bacteria or to Test for Presence of Bacteria in Purification Procedure

1. Gelatin 12% (Difco) - vitamins and peptones (Carmichael and Gorham, 1974).
2. Sodium Caseinate Agar - Taylor, C. B. 1940. J. Hygiene, 40:616.
3. Plate Count Agar (Difco) - liquid and solid.
4. Sodium carboxymethylcellulose (3%).
5. Lochhead's Soil Extract Semi-Solid Medium - Lochhead, A. G. and Margaret O. Burton. 1955. Can. J. Microbiol., 1:319.
6. Ascorbic Acid Broth (Difco) in ASM-1-TR.
7. ASM-1 plus vitamins and peptones (Carmichael and Gorham, 1974) - plus 2% soil extract.
8. Potato Dextrose Agar (Difco).
9. Maize Agar (Difco).
10. Casitone Agar - substitute Casitone (Difco) for Sodium Caseinate in Sodium Caseinate Agar.
11. Brewer Thioglycollate Medium (Difco).
12. Tryptone Broth (Difco).
13. Azotobacter Medium (Difco).
14. Skim Milk Medium - skim milk 2.5 g, yeast extract 0.25, sodium acetate 0.10 g - per 500 ml water. If for plates use 7.5 g agar. From F. D. Cook, Department of Soil Science.
15. Penassay Broth (Difco) - for nitrate and nitrite add 3 to 4 drops per culture tube of 5% solution.

APPENDIX III (cont.)

16. Board and Holdings Medium - $NH_4H_2PO_4$ 0.5 g, K_2HPO_4 0.5 g, yeast extract 0.5 g, agar 5.0 g per liter, Bromothymol Blue pH 7.2 (to color), and sterilized glucose 0.5% w/v. From Board, R. G. and A. J. Holding. 1960. J. Appl. Bact. 23(XI). This media replaces Hugh and Liefson Oxidation-Fermentation Test Medium (Edwards and Ewing, 1972).

APPENDIX IV

Animal Saline Solutions Used for Pharmacology Experiments

A. Animal saline:

9 g/liter NaCl in glass-distilled water. Used for routine IV injections of any compound. When used for flushing cannulas 100 IU/ml Heparin in the saline is used to prevent blood clots.

B. Krebs saline for mammalian or avian skeletal tissue - glass-distilled water:

Compound	Amount (g) for 10 liters
NaCl	69.0
KCl	3.5
$MgSO_4 \cdot 7H_2O$	2.9
KH_2PO_4	1.6
Glucose	20.0
$NaHCO_3$	21.0
$CaCl_2 \cdot 2H_2O$	4.4
Aerating gas	95% O_2 + 5% CO_2

C. Frog-Ringer saline for frog skeletal muscle - glass-distilled water:

Compound	Amount (g) for 10 liters
NaCl	65.0
KCl	1.4
NaH_2PO_4	0.6
Glucose	20.0
$NaHCO_3$	4.0
$CaCl_2$	10.8
Aerating gas	room air

APPENDIX V

Procedure for Recording Respiration, Arterial Blood Pressure, ECG, Heart Rate and Peripheral Muscle Contractions in the Anesthetized Rat

Rats, of either sex, weighing 300 to 500 g were used. Animals were anesthetized with an intraperitoneal injection of urethane (25%, 0.5 ml/1,000 g). The hair over the ventral surface of the neck was removed with clippers. A longitudinal incision was made from the larynx to the manubrium. The skin was divided and the connective tissue and salivary glands separated to expose the sternohyoideus muscles. These were divided to expose the trachea. The trachea was cleared of vascular connective tissue and a cannula, made from PE 240 (Intramedic (R)), inserted through a transverse incision and tied into place. The cannula was connected to a Grass volumetric pressure transducer for recording respiration. Artificial respiration, when done, was from a Harvard small animal respirator at 10 cc/stroke and 30 cycles/minute.

A carotid artery was located either side (usually right) of the trachea. It was separated from the vagus nerve which is connected to it. The vagus nerve can then have a stimulating electrode attached to it for tests on cardiac arrest. The cephalic end of the carotid artery was tied off and a cannula, made from PE 50 (Intramedic) inserted and tied into place. The cannula was kept filled with heparinized saline and attached to a P23Dd transducer.

ECG was recorded from the equivalent of human Lead II (Owen, 1973). These leads were to the four limbs (right hind leg ground) and a fifth to the central chest area. Heart rate was recorded using an HP

APPENDIX V (cont.)

model 8812A rate computer from either the pulse pressure or ECG trace as a trigger.

In some experiments the femoral artery was cannulated for blood pressure measurements. Both the femoral artery and vein come to a superficial position on the medial surface of the thigh along the boundary between adductor and extensor muscles. They are thus easy to locate and have ample length exposed for cannulation. Once they are located the procedure for cannulation is the same as with the carotid.

Intravenous (IV) injections of drugs were done using either the external jugular or femoral vein. They were cannulated with PE 20 (Intramedic) tubing.

The anterior tibialis muscle/sciatic nerve preparation for monitoring peripheral muscle contractions was obtained in the following manner. This muscle has its origin on the lateral side of the tibia. Its tendon passes under the annular ligament to the medial surface (inside of the leg) and attaches to the first metatarsal. The tendon was cut at its insertion and attached to a Grass force displacement transducer by a string. The sciatic nerve was located by a longitudinal incision on the posterior end of the thigh. The nerve lies deep between two longitudinal muscles, the heads of the gastrocnemius, lateral and media. The nerve was crushed centrally and an electrode hooked around it for square-wave stimulation of one per two seconds (5 volts).

Anatomical reference for this procedure is Green (1935).

APPENDIX VI

Procedure for Obtaining and Maintaining Rat Phrenic Nerve-Hemidiaphragms *in vitro*

This preparation was used for studying the effects of the toxin on twitches of a striated muscle that could be put up in an *in vitro* manner. The rats were killed by a blow to the head and bled from the neck. Since there are two phrenic nerves, one on each side of the chest, and the diaphragm can be cut down the middle giving two preparations, both phrenic nerves and the whole diaphragm were dissected. Skin and chest muscle were dissected away from the area of the thorax and the ribs cut through alongside the sternum. The ribs were cut around in an oblong shape towards the animal's flank. The upper part of the thorax was removed and the phrenic nerve was seen to run from the diaphragm up to the thymus gland. An incision was made in the abdominal wall just below the diaphragm and two cuts were made through the diaphragm. The ribs were left attached to the diaphragm on the spinal side. This gave a fan-shaped hemidiaphragm. The nerve was cut near the thymus and most connective material left attached. The preparation was placed in Krebs saline aerated with 95% CO_2, 5% O_2 at 37^{o}C. The apex of the preparation had some tendonous material which had a thread attached for connection to a Grass force displacement transducer. The base had some rib material left and this had a thread attached for connection to a holder. A 200-ml

APPENDIX VI (cont.)

organ bath was used for these preparations. The nerve was put through a tunnel electrode for stimulation of one pulse per two seconds at three volts.

The procedure was adapted from Perry (1968) p. 30.

APPENDIX VII

Preparation of Frog Rectus Abdominus Muscle for *in vitro* Muscle Contractions

Even though this muscle is striated it responds to acetylcholine by giving a slow contracture (tonic) rather than a fast contracture (twitch). It is, therefore, commonly used to show actions of those compounds which block transmission at the neuromuscular junction by acting in the same way as an excess of acetylcholine *e.g.* depolarizing drugs. Although these compounds will block transmission in the rat diaphragm they will clearly be seen as stimulating the slow fibers of the frog rectus.

Rana pipiens (approximately 20 g) were used. The frog was killed by a blow to the head and pithed. The skin was removed from the abdomen in an area from the sternum to the fork (junction of legs and body). The two rectus muscles were seen to run on each side of the midline from the base of the sternum to the fork. The sternum was cut through just above the xiphisternum at its base. The muscles were dissected out and placed in a petri dish (15 X 100 mm) containing frog-Ringer solution at room temperature. The muscles were divided at the midline and attached top and bottom to threads. The bottom thread was tied to a metal holding rod. The top thread was attached to a force displacement transducer having a spring attachment to give isotonic contractions of 1 g/cm. The muscle preparation was placed in a 20-ml organ bath containing frog-Ringer at room temperature aerated with room air, Perry (1968) p. 38.

APPENDIX VII (cont.)

The experimental dosing procedure was as follows:

1. Time 0: Ach or toxin extract added.
2. 2-min: The organ bath was drained.
3. 3-min: The bath was drained again.
4. 6-min: The next dose was given.

APPENDIX VIII

Procedure for Recording Peripheral Muscle Contractions from the Gastrocnemius Muscle of the Mallard Duck

Male mallard ducks weighing 800 to 1,000 g were anesthetized with 25 mg/kg of sodium pentobarbitol (Nembutal (R)) intravenously. The injection was given in one of the small veins located in the webbing of the foot or into a vein of the tarsus. The dose given was important because the usual dose for anesthetizing animals, *c.* 50 mg/kg, caused respiratory and cardiac arrest. The neck of the duck was held upright to avoid liquid draining into the lungs. The proper level of anesthetic was determined when the nictitating membrane failed to close when the eye was touched and when the pain reflex of the foot was gone. Trachea cannulation was done immediately after anesthesia because cardiac arrest and respiratory failure was found to occur unexpectedly in this species. This sensitivity to anesthetization may be involved with the diving reflex which ducks possess. When respiratory control was secured (a Harvard model 607 respirator at 150 cc/stroke and 18 cycles/minute was used when artificial respiration was necessary) the right jugular vein was cannulated, using PE (Intramedic) 50 or 90.

The sciatic nerve was located by a longitudinal incision (approximately 3 cm) in the posterior thigh. It lies deep and was seen by separating the semimembraneous and semitendonous muscles. A stimulating electrode was attached to the nerve with contractions

APPENDIX VIII (cont.)

formed by square-wave stimulations of one pulse per two seconds (15 volts). The nerve was crushed centrally. The transducer string was attached to the ligament of the gastrocnemius pars externa. The ligament was located by a small incision at the back of the tarsus near the foot. The thigh was clamped in a rigid position to avoid movement of the leg. The anatomy references are from (Simons, 1960; and Berger, 1960).

APPENDIX IX

Preparation of the Isolated Guinea-Pig Ileum for *in vitro* Muscle Contractions

The guinea-pig was killed by snapping the spinal cord of the neck. The throat was cut to bleed the animal. The abdomen was opened and the ileum located where it joined on the back of the caecum. About a 10 cm piece was removed and placed in Krebs saline. Since the animal was starved for 24 hours it was not necessary to clean the piece of ileum. Approximately 3-cm pieces were used for the experimental test. The mesentery was removed from the piece and thread attached to the top and bottom of the muscle. The muscle piece was attached to the transducer in the same manner as with the frog rectus. A 20-ml organ bath was used that contained Krebs saline at 37°C aerated with 95% O_2, 5% CO_2, Perry (1968) p. 58.

Contractions in response to added compounds were recorded from a force displacement transducer with a 1 g/cm spring attachment. The experimental dosing procedure was as follows:

1. Time 0: Dose added.
2. 20-seconds: Wash with Krebs saline.
3. 60-seconds: Wash with saline.
4. 2-minutes: Add next dose.